BIOCHEMICAL STUDIES ON PROTOZOA

by

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T O M Y P A R E N T S A N D U N A

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Introduction

Protozoa are defined in the elementary text-books as simple unicellular animals. However, many of them synthesise chlorophyll and starch, which are characteristic of plants, although in other respects they may resemble animals. The higher organisms can readily be divided into two kingdoms, plant and animal, but this is not always possible with the more primitive forms of life and inevitably some overlap occurs in classification. For example, Euglena gracilis is considered by Jahn (1949) as a protozoan, but by Fogg (1953) as an alga. Attempts to divide the primitive forms of life into plants and animals by consideration of their methods of nutrition or by motility have also met with difficulty, and it is now generally recognised, that it is not easy to give a complete and accurate definition of a protozoan (see for example Lwoff, 1951 and Jahn, 1949).

Protozoa are unicellular and possess at least one nucleus. They are divided into four main groups, three of which are distinguished by their means of locomotion and the members of the fourth group differ from the others in that they reproduce by means of spores.

The first group move by means of pseudopodia. A pseudopodium is an elongate extension of the protoplasm and movement of the organism may result from a true protoplasmic flow as in Amoeba proteus or in some species (such as the shelled amoeba Arcella) the front end of the pseudopodium becomes attached to the surface, on which the organism is resting, prior to contraction, thereby

pulling the rest of the organism.

The second group of protozoa (the ciliates) move by means of small hair-like structures known as cilia. Each individual cilium has a power stroke, during which it is quite rigid and it then becomes flexed during the return stroke. Generally the cilia do not beat in phase; some are in the power stroke while others are in the return stroke, thus producing a characteristically smooth movement.

In contrast to this, the third group (the flagellates) move in a rather erratic manner; their mode of locomotion is by means of flagella. The flagellum may consist of a simple whip-like fibre, as in Trichomonas, or it may have small projections (mastigonemes) attached to it. In either case, the organism is propelled by a wave motion passing along the flagellum.

The mode of reproduction in protozoa is highly variable among the different groups, although it is primarily one of cell division (Kudo, 1954). Reproduction is initiated by nuclear division in nearly all cases. In most flagellates, binary fission occurs by longitudinal division of the cell from the anterior end. In most of the ciliates binary fission is transverse.

The many species of protozoa live under diverse conditions and obtain their food in a variety of ways. Protozoa may be free living, i.e. they live in ponds, streams, soil or other similar places, or they may be dependant on other organisms for their existence. This latter group are divided into two categories: (a) protozoa which form a symbiotic relationship with the host

organism, resulting in mutual benefit (for example, the rumen protozoa found in sheep, cattle and other ruminants); (b) the parasitic protozoa, which live on or in another organism, resulting in disease in the host.

Among the more familiar protozoal diseases, which occur in man, are malaria (caused by Plasmodium viva, P.malariae, P.ovale and P.falciparium), amoebic dysentery (caused by Endamoeba histolytica) and sleeping sickness (caused by Trypanosoma gambiense)(for a review see Goodwin and Rollo, 1955). Parasitic protozoa also inhabit animals causing diseases, which in many cases prove fatal (Morgan and Hawkins, 1948). For example, Amoeba meleagridis causes blackhead in poultry and Piroplasma bigeminum causes piroplasmosis (more commonly known as bovine malaria) in cattle. The medical, veterinary and economic importance of diseases caused by protozoa has led to the development of protozoacidal drugs and to a considerable amount of research on the metabolism of these parasites.

There are three basic ways by which protozoa may acquire food. Holozoic nutrition is the method used by higher animals and by several protozoa. It involves capture of particulate food followed by ingestion, digestion, assimilation and rejection of indigestible portions. The methods of food capture vary among different forms. In the amoeba, pseudopodia are formed which surround particulate food and draw it towards the main body of the organism. In certain of the Mastigophora, the flagellar movement draws the food particles to the cytostome where they

enter the body of the organism. Chen (1950) observed Peranema feeding on immobile organisms. When the tip of the anterior flagellum comes in contact with an immobile Euglena, the whole flagellum beats actively and the body contracts, followed by elongation. The process is repeated several times until the body touches Euglena. The cytostome stretches open and the whole Euglena is engulfed.

Many flagellates possess photosynthetic pigments and are thus able to utilise carbon dioxide and inorganic salts to satisfy their nutritional requirements. This is known as holophytic (or phytotropic) nutrition. Some photosynthetic protozoa, however, are able to produce pseudopodia at certain phases of their lives and can then ingest particulate food.

The third method, by which protozoa may obtain food, is known as saprozoic nutrition. This involves diffusion through the body surface and does not require any special organellae.

Many protozoa nourish themselves by more than one method at the same or different times. For example Euglena gracilis loses its green coloration in the dark or even in the light when the culture medium contains a large amount of organic substances, which may indicate that this organism is capable of carrying on both holophytic and saprozoic nutrition (Pringsheim and Hovasse, 1948).

The digestion of food by protozoa results in growth and increase in volume of the organism, and also in the formation and storage of reserve materials, which are deposited in the cytoplasm

to be utilised later for growth or reproduction. Glycogen has been identified as the reserve material in many species, by iodine staining methods (see for example, Dutta, 1960). Protozoa, which utilise food by holophytic nutrition, may synthesise starch or paramylon as reserve polysaccharides in addition to oil and fats. The Chrysomonadina store oil and a water-soluble carbohydrate known as leucosin, whereas the rumen protozoa generally synthesise amylopectin.

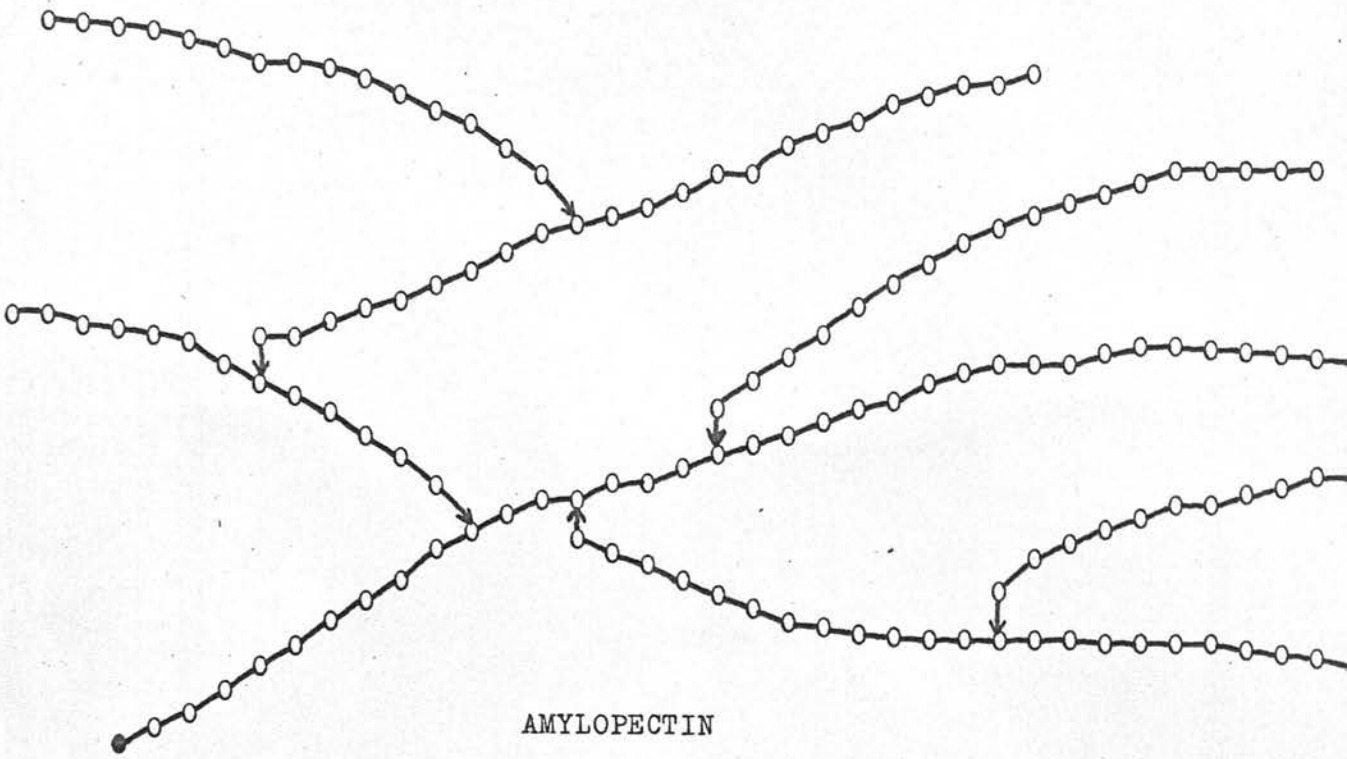
The main part of the present investigation is concerned with chemical examination of polysaccharides synthesised by Entodinium caudatum, Polytoma uvella, Ochromonas malhamensis, Peranema trichophorum and Astasia ocellata.

The latter part of the present work is concerned with studies on the specificity of transglycosylase activities present in cell-free extracts of Tetrahymena pyriformis GL and on the synthesis of oligosaccharides by this enzyme system.

C H A P T E R 1

α-GLUCANS FROM PROTOZOA

BASIC STRUCTURES OF SOME α-GLUCANS



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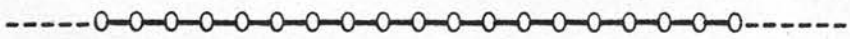
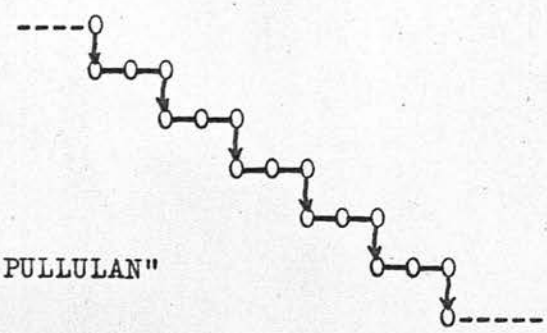


AMYLOSE



"PULLULAN"

DEXTRAN

Key:- ——, α-(1→4)-linkage.

→, α-(1→6)-linkage.

O , glucose residue.

FIGURE 1

Nature and Occurrence of α-Glucans

α-Glucans are widely distributed in Nature; they occur as reserve polysaccharides in plants and animals and are synthesised extracellularly by moulds and bacteria. They may also occur as cell wall material (Ralph, Bender, Leis and O'Brien, 1961). The mode of linkage of the glucose units, the degree of branching and the molecular weight of α-glucans vary considerably. For example, nigeran contains α-(1→3) and α-(1→4)-linkages (Barker, Bourne and Stacey, 1953) whereas dextran contains mainly α-(1→6)-linkages. "Pullulan," an extracellular glucan from Pullularia pullulans, is composed almost entirely of maltotriose units linked by α-(1→6)-glucosidic linkages to form a linear chain (Bender, Lehmann and Wallenfels, 1959, and Bender and Wallenfels, 1961) (see figure 1).

Since all protozoal α-glucans so far examined are of the starch, glycogen or amylopectin-type the present discussion is confined to these latter polysaccharides.

Starch.

Starch forms the principal food reserve in many plants; it occurs as discrete granules which vary in size depending on the source and on the maturity of the plant (Banks and Greenwood, 1959). Most starches contain two components, amylose and amylopectin in the ratio of about 20:80. However, the waxy cereal starches contain little if any amylose (Bates, French and Rundle, 1943) whereas certain varieties of maize starch contain as much as 50% amylose (for review, see Aspinall and Greenwood, 1962).

Amylose.

Amylose is an essentially linear polymer of D-glucose units linked by α-(1→4)-glucosidic linkages. Peat, Pirt and Whelan (1952) showed that amylose was hydrolysed to maltose to the extent of only 68-70% by highly purified β-amylase, thus suggesting the presence of anomolous linkages. Kjölberg and Manners (1963) have shown that the barriers to β-amylase are most probably a small proportion of α-(1→6)-glucosidic interchain linkages.

Amylopectin.

Amylopectin consists of chains of D-glucose units linked by α-(1→4)-glucosidic linkages, which are interlinked by α-(1→6)-glucosidic linkages in a random fashion to form a branched structure. Most amylopectins contain 4-5% of interchain linkages, which represents on a statistical basis an average chain length of 25-20 glucose residues.

Glycogen.

Glycogen is the characteristic reserve polysaccharide of animals, although it also occurs in protozoa, bacteria and yeast. Glycogen, like amylopectin, consists of chains of α-(1→4)-linked glucose units interlinked by α-(1→6)-interchain linkages. It differs from amylopectin, in that the chains are much shorter, thus forming a more compact, bush-like structure. The average chain length is generally in the region of 10-16.

α-Glucans from Protozoa.

By iodine staining methods, starch has been tentatively identified as the reserve polysaccharide of several green and colourless flagellates (Hunter and Provasoli, 1951). Similarly, glycogen has been recognised in several "animal-like" ciliates by its brown coloration with iodine (see, for example, Dutta, 1960), whereas the reserve material of holotrichously ciliated protozoa from the rumen of sheep, stains purple with iodine, suggesting the presence of amylopectin (Oxford, 1951).

The presence of a starch-type polysaccharide was indicated in Polytoma obtusum (Brechot, 1937). The material stained blue with iodine and was degraded by amylases, releasing a substance which reacted with Fehling's solution. A thorough characterisation of starch granules from Polytomella coeca was carried out by Bourne, Stacey and Wilkinson (1950). The material closely resembled plant starch but had a slightly lower amylose content (13-16%, compared with 20-25% in potato starch). The starch-type polysaccharide from Chilomonas paramecium also resembles plant starches but has an amylose content of 45% (Archibald, Hirst, Manners and Ryley, 1960).

Reserve polysaccharides, which closely resemble animal glycogen, have been isolated from Tetrahymena pyriformis (Manners and Ryley, 1952) and from Trichomonas foetus and Trichomonas gallinae (Manners and Ryley, 1955) although the polysaccharides from the latter two organisms differed slightly from animal glycogen in their branching characteristics.

An amylopectin-type polysaccharide has been isolated from a mixture of holotrichously ciliated rumen protozoa and characterised by methylation and periodate studies (Forsyth and Hirst, 1953); this has been verified by enzymic studies (Mould and Thomas, 1957). An amylopectin-type polysaccharide also occurs in Cycloposthium, a protozoan found in the colon and caecum of the horse (Forsyth, Hirst and Oxford, 1953).

Polysaccharide Metabolism in Protozoa.

Although the biochemical pathways for synthesis and breakdown of starch and glycogen in plants and animals are basically established (for a review, see Manners, 1962), little is known of the metabolism of protozoal polysaccharides. Protozoal polysaccharides may originate from acetate, alcohols, lipids or from simple sugars (Manners and Ryley, 1963) and in several cases the end-products of polysaccharide fermentation (generally low molecular weight fatty acids) have been examined (for example, Howard, 1963). The presence of phosphorylase activity has been shown in Tetrahymena pyriformis (Ryley, 1952), rumen protozoa (Mould and Thomas, 1957) and in Polytomella coeca (Lwoff, Ionesco and Gutmann, 1950). Bebbington, Bourne, Stacey and Wilkinson (1952) showed that Polytomella coeca also possesses an enzyme, similar to plant Q-enzyme, which would convert amylose into an amylopectin-type polysaccharide. They suggested that amylose is synthesised by successive transfer of glucosyl radicals from α-D-glucosyl phosphate. Some of the linear chains of α-(1→4)-linked glucose /

units are then converted into amylopectin under the action of Q-enzyme. However, the role of phosphorylase in starch and glycogen metabolism is now believed to be one of degradation rather than synthesis (Manners, 1962). Leloir and his co-workers (1957, 1959, 1960) have shown that glycogen can be synthesised by a transglucosylation reaction in which uridine 5-(D-glucosyl pyrophosphate) (UDPG) rather than α-D-glucosyl phosphate acts as D-glucosyl donor. The related transglucosylase starch-UDP glucosyl transferase has now been shown in extracts of potatoes, sweet corn and beans (Leloir, de Fekete and Cardini, 1961).

Up to the present, UDPG has not been implicated in the synthesis of α-glucans by protozoa, although Goldemburg and Marechal (1963a) have shown that it may be involved in the synthesis of paramylon granules by Euglena gracilis.

SECTION (B)

Experimental Methods

1. Drying of Polysaccharide Samples.

The material was washed successively with ethanol and ether and dried at 60° *in vacuo* over phosphorus pentoxide for not less than 12 hr.

2. Evaporations.

All evaporations were carried out under reduced pressure at temperatures not exceeding 40°.

3. Estimation of Reducing Sugars.

For estimation of glucose and maltose, the method of Somogyi (1952) was used. Calibrations using glucose (0·2-1·2 mg.) and maltose (0·4-2·4 mg.) were made on solutions determined polarometrically after allowing mutarotation to proceed to equilibrium, using $[\alpha]_D^{20}$ = +52·6° for glucose and +138° for maltose. The ratio, mg. sugar: titre difference from blank (in ml. 0·01N-sodium thiosulphate) was generally about 0·29 for glucose and 0·52 for maltose; slight differences in these ratios were noted with different batches of the reagent. The reagent was kept at 37° to prevent crystallisation.

4. Paper Chromatography.

All chromatograms, in this chapter, were run on Whatman No.1 paper and developed in ethyl acetate - pyridine - water (10:4:3, by vol.) (abbreviated, throughout the text, to 10:4:3).

The dried chromatograms were sprayed with a saturated solution

of aniline oxalate in methylated spirits (Partridge, 1949) and heated at 140° for 3-4 min. The best colours (pink with pentoses and yellow with hexoses) were obtained if the chromatogram was then exposed to daylight for 30 min.

Silver nitrate spray reagent (Trevelyan, Procter and Harrison, 1950) was applied, either to the aniline oxalate treated chromatogram (producing a permanent record and a slightly increased sensitivity) or to the dried chromatogram. The treatment with silver nitrate involved three solutions:-

A. To acetone (200 ml.) was added saturated silver nitrate solution (1 ml.), followed by the dropwise addition of water until the precipitate just redissolved.

B. 1ON-NaOH (10 ml.) mixed with methylated spirits (90 ml.).

C. Saturated aqueous solution of sodium thiosulphate.

The dried paper was dipped in solution A, allowed to dry, sprayed with solution B, heated for 20 sec. at 80°, dipped in solution C and washed for 2-3 hr. in water.

5. Estimation of Methylated Sugars.

The estimation of methylated sugars was carried out by the method of Schaefer and Van Cleve (1956). Standard solutions of 2,3-di-O-methyl-D-glucose; 2,3,6-tri-O-methyl-D-glucose; and 2,3,4,6-tetra-O-methyl-D-glucose were prepared and spotted on the starting line of chromatograms using 5 and 10 µl pipettes. The chromatograms were developed in methyl ethyl ketone - water - ammonia (200:17:1, by vol.) using 2 descents (4 hr. each). The methylated sugars were located by means of control strips and the

sugar-bearing areas cut out, attached by a small wire to the foot of a condenser and extracted by refluxing a solution containing 1·00% (w/v) tetraethylene glycol dimethyl ether in methanol (5 ml.) for 20 min. To the solution was added a 2·4% solution of aniline hydrogen phthalate in methanol (1 ml.) and the methanol removed by evaporation under reduced pressure at 30°. The residual pool of liquid was heated at 98° for 35 min., cooled and then dissolved in commercial ethanol (10 ml.). The absorbance of the sugar solutions was measured in a Unicam S.P.500 Spectrophotometer at 415 mμ against ethanol as blank. Corrections were made by running "paper blanks" through the above procedure. In some initial experiments, ethylene glycol was used instead of tetraethylene glycol dimethyl ether. The results were similar but not so consistent.

For determination of the ratio of methylated sugars from the hydrolysed methylated polysaccharide, the mixture was treated in the same way as the standard solutions but the trimethylglucose fraction had to be diluted. All such dilutions were carried out using the eluting solvent in order to provide the standard amount of tetraethylene glycol dimethyl ether in the 5 ml. aliquots upon which the determinations were performed. Dilutions were not carried out after the development of colour. The calibration values were tetramethylglucose 266, trimethylglucose 370, dimethylglucose 484.

6. Total Acid Hydrolysis.

(a) For qualitative experiments, the solid material (1 mg.)

was heated with 2N-H_2SO_4 (2 drops) at 100° for 2 hr. Neutralisation was carried out using barium carbonate.

(b) For quantitative experiments, 4N-H_2SO_4 (1 ml.) was added to an aqueous solution of the α-glucan (0·5-1·7 mg. in 1 ml.) and the mixture heated at 100° for 2·25 hr. The hydrolysate was neutralised with 2N-NaOH (phenolphthalein) and made very slightly acidic with 0·1N-H_2SO_4. The glucose produced was estimated with Somogyi reagent.

7. Determination of β-Amylolysis Limits.

All experiments with β-amylase were carried out using a Wallerstein Laboratories (New York) preparation, which was devoid of maltase activity but possessed very small traces of α-amylase (Z-enzyme). The activity of the enzyme preparation was 100 units/mg. as determined by the method of Hobson, Whelan and Peat (1950). The maltose liberated was estimated by Somogyi reagent.

8. Blue Value.

The "blue values" of starch, amylopectin and amylose were determined under the conditions of Bourne, Haworth, Macey and Peat (1948). The volume of solution containing exactly 1 mg. polysaccharide (estimated by acid hydrolysis and glucose determination) was transferred to a 100 ml. standard flask, 6N-HCl (0·05 ml.) and about 20 ml. of water added. A solution containing 0·2% iodine and 2% potassium iodide (1 ml.) was pipetted in and the solution diluted to 100 ml. The absorption was read, against the corresponding iodine blank, in 2 cm. cells at 680 mμ using a Unicam S.P.600 Spectrophotometer and the reading doubled to give

the "blue value."

9. λmax of the Glucan-Iodine Complex.

For amyloses and starches the λmax was determined using the "Blue Value" solutions, readings being taken from 450-700 mμ. For amylopectins the λmax was obtained using solutions which were five or ten times as strong as the "Blue Value" conditions, but the ratio of iodine:polysaccharide was kept the same.

10. Potentiometric Iodine Titrations.

The method was essentially that of Bates, French and Rundle (1943). Starch (30-50 mg.) or amylose (8-15 mg.) or amylopectin (60-100 mg.) was dissolved by magnetic stirring in 0·5N-KOH (5 ml.) for 4-5 hr. The solution was neutralised, to methyl orange, using 0·5N-HCl, and 0·5N-potassium iodide (10 ml.) added. The solution was diluted to exactly 100 ml. with distilled water and titrated at room temperature (18°) with a standard iodine solution of normality ca.0·001N, which was 0·05M with respect to potassium iodide. The electrical potential of the solution was measured, after each 1 ml. addition of iodine solution, using a bright platinum electrode in the solution connected by a salt bridge to a calomel electrode (saturated with respect to potassium chloride and mercurous chloride). The electrodes were connected to the millivolt terminals of a Pye Universal pH meter. The solution was stirred for about 3 min. after each addition of iodine solution to allow equilibrium to be established between the polysaccharide and the iodine. A "blank" experiment was carried out, exactly as described above, except that the polysaccharide was omitted.

Method of Calculation:- Consider the addition of iodine solution (10 ml.) to the "blank" experiment (B) and to the starch experiment (S). The difference in e.m.f. between B and S, (E_B-E_S) will be the same as the e.m.f. produced in a concentration cell experiment with B and S as the electrodes.

Hence $E_B-E_S = \frac{RT}{nf}\ln[I_2]_B - \frac{RT}{nf}\ln[I_2]_S$

$$\ln\frac{[I_2]_B}{[I_2]_S} = \frac{nf}{RT}.\Delta E$$

Now $I_2 + I^- + 2e \rightarrow I_3^=$ (i.e. n = 2)

and $\frac{RT}{f} \times 2.303 = 0.05775$ at 18°C

$$\frac{[I_2]_B}{[I_2]_S} = \text{Antilog } \frac{\Delta mV}{28.87}$$

Where

n = No. of faradays.

f = one faraday.

$[I_2]_B$ and $[I_2]_S$ are the concentrations of iodine in moles per litre in the "blank" and "starch" experiments respectively.

T = Temperature in degrees absolute.

mV = millivolts.

$[I_2]_B$ is known from the amount of iodine added and the total volume of the solution. Hence $[I_2]_S$ can be found. $[I_2]_B-[I_2]_S$ gives the value of iodine "bound" to the starch in moles per litre. The "bound" iodine is generally expressed in mg. iodine per 100 mg. starch. By plotting the concentration of "free" iodine against the "bound" iodine and extrapolating to zero free iodine concentration, the "iodine affinity" can be found.

With certain samples, the amount of material was insufficient to carry out the procedure as described above. In such cases, the

quantities were divided throughout by a factor of twenty, keeping the concentrations the same as before. The solution was stirred by a small magnetic stirrer and a microburette was used to make 0·05 ml. additions of iodine solution. Using this modified procedure, care had to be taken to ensure that the solution from the calomel electrode did not leak over into the polysaccharide solution.

11. For comparative purposes potato, oat and maize starches were prepared and fractionated into their amylose and amylopectin components. The extractions and purifications were carried out by the methods described by Cowie and Greenwood (1957) for potato starch and Anderson and Greenwood (1955) for the oat and maize starches. The starches were fractionated by the method described by Cowie, Fleming, Greenwood and Manners (1957).

SECTION (C)

STUDIES ON THE RESERVE POLYSACCHARIDE FROM ENTODINIUM CAUDATUM

Introduction

Entodinium caudatum is a ciliate protozoan found only in the rumen. Rumen ciliate protozoa can be divided roughly into three groups both according to their body form and their metabolic activities. There are the Holotrichous ciliates (holotrichs), and two groups of Oligotrichous ciliates (oligotrichs), the large and the small types. The holotrichs are so called because their bodies have a uniform covering of small slender cilia. The oligotrichs, on the other hand, are characterised by one or two specialised groups of cilia at the anterior end of the organism. Oligotrich ciliates from rumen ingest plant particulate matter but unlike the holotrichs are apparently unable to utilize soluble sugars (Oxford, 1955). Entodinium caudatum belongs to the group of small oligotrich protozoa; it measures 50 to 70μ in length by 30 to 50μ in breadth.

The genus Entodinium was established by Stein in 1858 for several species of ciliate occurring in the stomach of ruminants. The body is ovoid in shape, while the anterior end has a spiral row of cirri leading to the oesophagus. The posterior end of the body is prolonged into several caudal processes. There is an elongate macronucleus extending along one side of the body and a

small micronucleus. One or more contractile vacuoles are present.

The function of protozoa in the rumen has been a matter of controversy, but it is now believed that there is a symbiotic relationship, from which both protozoa and ruminant benefit, (Sugden, 1954). The diet of ruminants consists largely of enzymically resistant carbohydrates cellulose and hemicellulose; the proportion of soluble, easily digestible sugars is normally very small. The ruminant itself does not possess the necessary enzymes to utilise these carbohydrates, but the micro-organisms (bacteria and protozoa) present in the rumen perform this function. For example, extracts of Dasytricha ruminantium, Isotricha intestinalis and Isotricha prostoma possess pectin esterase and polygalacturonase activities (Abou Akkada and Howard, 1961). Epidinium ecaudatum, isolated from cows have been shown to contain amylase, maltase, hemicellulase (xylanase and arabinosidase) (Bailey, Clark and Wright, 1962) and pectinase (Wright, 1961). In contrast to this Entodinium caudatum appears to live solely on starch granules for its carbohydrate requirements (Hungate, 1943). This has been confirmed by Abou Akkada and Howard (1960) who showed that cell-free extracts of the organism possess strong amylase and maltase activities but only traces of other carbohydrases. These workers have also shown that the products of carbohydrate fermentation were volatile fatty acids (chiefly butyric acid and acetic acid, with a little propionic acid and formic acid), carbon dioxide, hydrogen and about 2% lactic acid. The amount of lactic acid produced is in contrast to the amount

produced by holotrichs, where about 50% of the carbon in the fermented sugar is converted to lactic acid.

The nitrogen metabolism of Entodinium caudatum has also been examined (Abou Akkada and Howard, 1962) but complete biochemical studies on both the carbohydrate and nitrogen metabolism are not possible at present since pure cultures cannot be grown in the laboratory. This is possibly because the large amounts of fatty acids produced make exact pH control difficult and the anaerobic conditions, necessary for survival, are difficult to maintain.

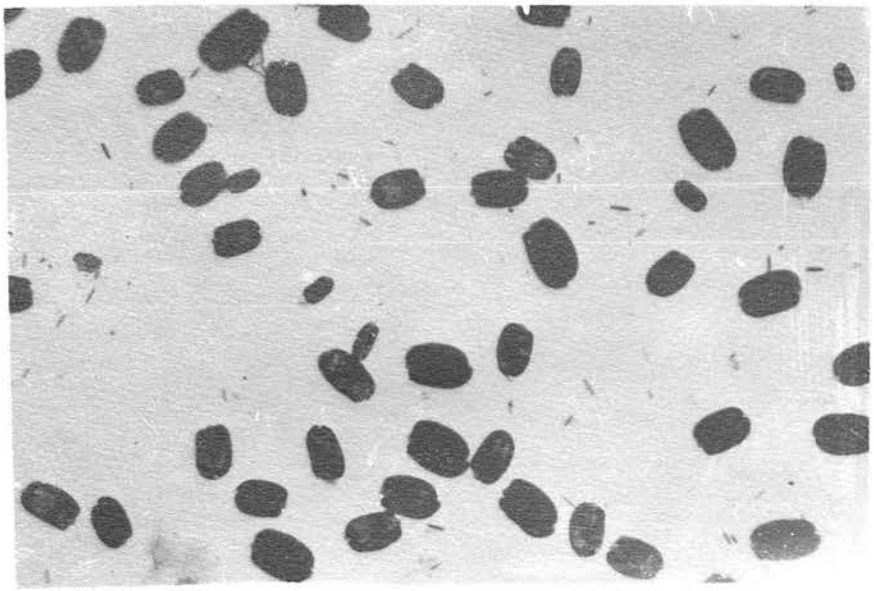
Recently, it has been suggested that Entodinium caudatum may be an atypical member of the genus Entodinium (Bailey and Clarke, 1963). When sheep were fed on pure rye-grass pastures nearly 80% of the protozoa were Entodinium species, but Entodinium caudatum was absent. Since rye-grass contains less than 0·7% (dry weight) of starch, it was concluded that Entodinium caudatum was the only species of the genus incapable of surviving on cellulose or hemicellulose material. In support of this, these workers have shown that the entodinia found under non-starchy conditions possess xylanase and other enzyme activities from which they can survive.

Entodinium caudatum metabolises starch granules but not starch given in solution. The starch granules pass into the gastric sack where they are converted partly into fatty acids and partly into reserve polysaccharide, which is deposited mainly round the tails, the edges of the cells and the mouth. If the organism is starved, the starch granules disappear and only the reserve polysaccharide remains. On continued starvation, the reserve polysaccharide can

be fermented by the organism.

The object of the present experiments was to isolate and characterise the reserve polysaccharide.

a



b

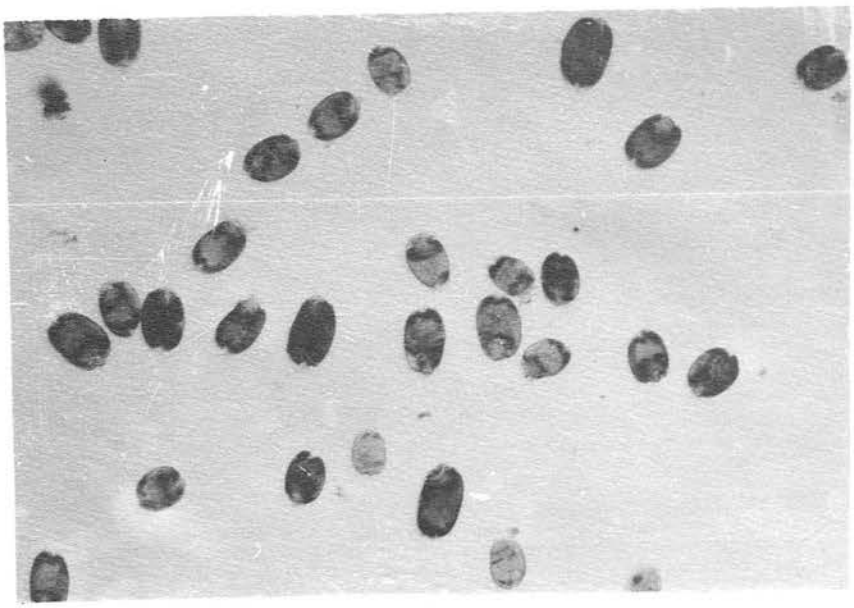


FIGURE 2

Entodinium caudatum

c

EXPERIMENTAL SECTION

Isolation of *Entodinium caudatum*

(Carried out at the Rowett Institute, Aberdeen, by Dr. J.M.Eadie).

Samples of rumen liquor were withdrawn from the single sheep (No.105) just before the 9.30 a.m. meal, so that the cells were starved at least 18 hr. ensuring that they were devoid of plant starch. The procedure of obtaining washed suspension of entodinia was essentially that described by Abou Akkada and Howard (1960). A suspension of *Entodinium caudatum* and *Dasytricha ruminantium* was obtained by separation and decantation and these organisms were kept for 12 hr. in buffer containing chloramphenicol (50 g./ml.). Treatment for a further 3 hr. in buffer containing 0·6% mannose killed all the *Dasytricha ruminantium*. The *Entodinium caudatum* were then washed and stored at -20° until required.

Figure 2 shows *Entodinium caudatum* stained with dilute Lugol's iodine. In (a) (magnification x 150) the cells are filled with plant starch whereas in (b) the cells have been starved and most of them are devoid of plant starch. Figure 2 (c) shows three empty cells at a magnification of 300. In the top left one, the cilia are extended; all three cells indicate clearly that the species is *caudatum*.

Methods of Extraction.

(a) Attempted extraction by the method of Clarke and Stone (1960).

Since the method of Clarke and Stone involves the use of trypsin, it was necessary to test the trypsin preparation for amylase activity. This was carried out as follows:-
Amylose (from potato starch var. Duke of Kent) (23.0 mg.) was dissolved in 0.1N-KOH (3 ml.) and neutralised with 2N-H_2SO_4 (phenolphthalein). 0.1M-Acetate buffer of pH 7.6 (5 ml.) was added and the solution allowed to come to 25°. To this was added trypsin (20 mg. of a Nutritional Biochemicals Corp. preparation in 5 ml. of 0.1M-acetate buffer of pH 7.6) and the solution placed in a modified Ubbelhode viscometer, where it was mixed by bubbling air through it gently. After allowing time for the solution to reach the temperature of the thermostat (25°), the time required for the liquid level to pass the two marks on the viscometer was measured. The solution was allowed to stand at 25° for 24 hr. and the time measured again.

Time (initially) = 2 min. 16.01 sec.

Time (after 24 hr.) = 2 min. 15.73 sec.

In addition, no change in iodine staining was observed during the incubation period.

A suspension of Entodinium caudatum cells was diluted with methanol and subjected to ultrasonic vibrations for 5 min. at 2°. The disrupted cell material was centrifuged down; the supernatant solution gave no colour with iodine. The residue was exhaustively

defatted with methanol, washed with ether and dried under vacuum over P_2O_5. The solid material (1·10 g.) was incubated at 37° with trypsin (0.50 g.) in phosphate buffer of pH 7.6 (30 ml.) and covered with a little toluene. After incubation for 2 days, the digest was centrifuged and the supernatant solution evaporated down to small volume and tested with iodine; only a feeble tinge was produced. The residue was washed several times with a saturated aqueous solution of urea.

Examination of urea washings. The solution was dialysed for 2 days against several changes of distilled water. During this time some material was spontaneously precipitated from solution. The material was largely insoluble after treatment with 2N-H_2SO_4 at 100° for 2 hr., but on neutralisation traces of glucose and galactose were detected by paper chromatography.

The solution remaining in the dialysis bag was freeze-dried and on dissolving in water it gave a brown solution, which masked colour tests with iodine. On total acid hydrolysis it had an apparent polyglucose content of 5·5% (Somogyi reagent) and on examination of the neutralised acid hydrolysate, glucose (++++), galactose (+), xylose (+), mannose (±) and another sugar of R_G value 1·75 (10:4:3 as solvent) were detected.

Examination of the residue from tryptic digestion. The residue was expected to contain the starch granules but it was dark brown in colour. The colour did not improve on further washings with urea solution and water. In an attempt to purify the material

it was put through a second treatment with trypsin. The residue (ca. 15 mg.) after this treatment did not show any improvement in colour, and on total acid hydrolysis it yielded glucose (++++), galactose (±), mannose (±) and xylose (±).

The method of Clarke and Stone was therefore unsuitable.

(b) Extraction with chloral hydrate.

It has been reported that the extraction of starch-type polysaccharides with chloral hydrate causes severe degradation (Anderson and King, 1961), although previous to this report it had been successfully used in the extraction of other protozoal polysaccharides (Bourne, Stacey and Wilkinson, 1950; Forsyth and Hirst, 1953). In view of this, the effect of chloral hydrate on amylose was investigated under aerobic and anaerobic conditions.

Amylose (ca.200 mg. of a sample prepared from potatoes, var. Duke of Kent) was dissolved in M-KOH (20 ml.) and the solution neutralised with 2N-H_2SO_4 (phenolphthalein). After addition of a 66% (w/v) aqueous chloral hydrate solution (30 ml.) the solution (which had a pH of 3·8) was treated with a steady stream of nitrogen and the temperature raised to 80°. These conditions were maintained for 1 hr. The solution (which now had a pH of 3·6) was dialysed against tap water for 2 days and the amylose precipitated with ethanol, washed successively with butanol, ethanol and ether and dried under vacuum.

The above process was repeated but air was bubbled through

the solution in place of nitrogen. Viscosity determinations were carried out on the original amylose and on the chloral hydrate treated amyloses, as follows:- amylose (100 mg.) was dissolved by gentle agitation in M-KOH (20 ml.). 3 ml. of this solution were diluted to 25 ml. with distilled water and portions (1 ml.) taken for acid hydrolysis and Somogyi estimations. M-KOH (10 ml.) was pipetted into the modified Ubbelhode viscometer and the time required for the liquid level to pass the two marks measured. Amylose solution (3 ml.) was added to the potassium hydroxide solution in the viscometer, mixed by bubbling nitrogen through it and the flow time taken again. This process was repeated using successive 3 ml. additions of amylose solution. All solutions were filtered through a G3 sinter glass and kept at 25° in the thermostat; time was allowed after each addition of amylose solution to ensure that temperature equilibrium had been attained. In each case ten readings were taken using a stop-watch and the mean value noted.

Results: Original Amylose

C	To	T	$\frac{T-To}{To}$	$\frac{1}{C}$
1.185	236.3	313.2	273	
1.927	236.3	368.4	290	
2.432	236.3	407.4	297	
2.807	236.3	431.8	304	

Where

C = concentration of amylose (mg./ml.)

To = flow time of solvent (secs.)

T = flow time of solution (sec.).

EFFECT OF CHLORAL HYDRATE ON AMYLOSE

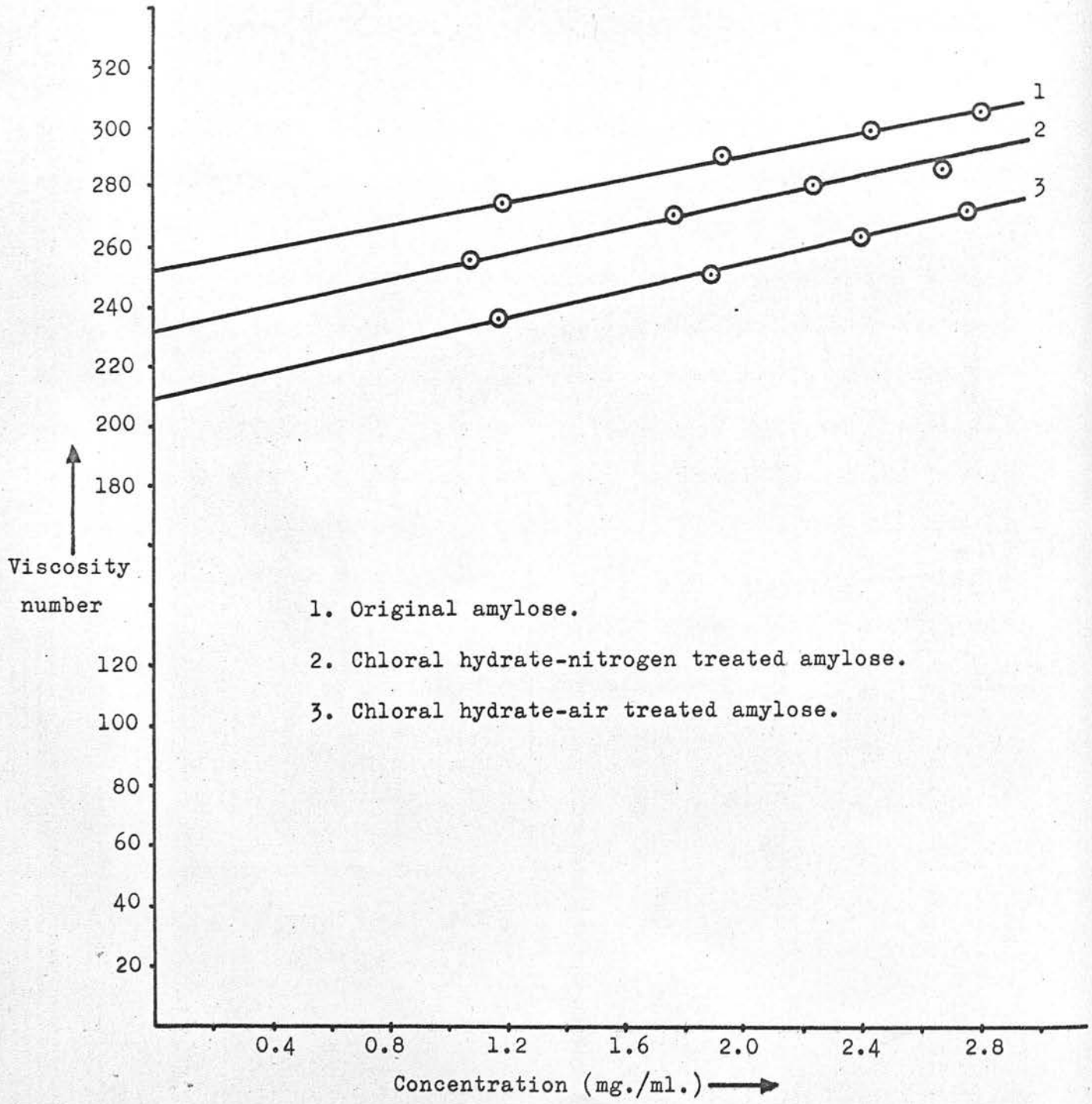


FIGURE 3

Chloral hydrate - air-treated amylose.

C	To	T	$\frac{T-To}{To} \times \frac{1}{C}$
1·165	126·2	160·9	236
1·894	126·2	186·1	250
2·391	126·2	205·3	262
2·757	126·2	220·5	271

Chloral hydrate - nitrogen-treated amylose

C	To	T	$\frac{T-To}{To} \times \frac{1}{C}$
1·091	126·2	161·1	253
1·774	126·2	186·9	270
2·241	126·2	205·4	280
2·580	126·2	219·2	285

$\frac{T-To}{To} \times \frac{1}{C}$ is known as the Viscosity Number and by extrapolation of the graph of Viscosity Number against concentration to zero concentration the Limiting Viscosity Number $[\eta]$ is obtained. Cowie and Greenwood (1957) have shown that for amylose the degree of polymerization (D.P.) is given by,

$$D.P. = [\eta] \times 7{\cdot}4$$

Hence D.P. of original amylose = 250

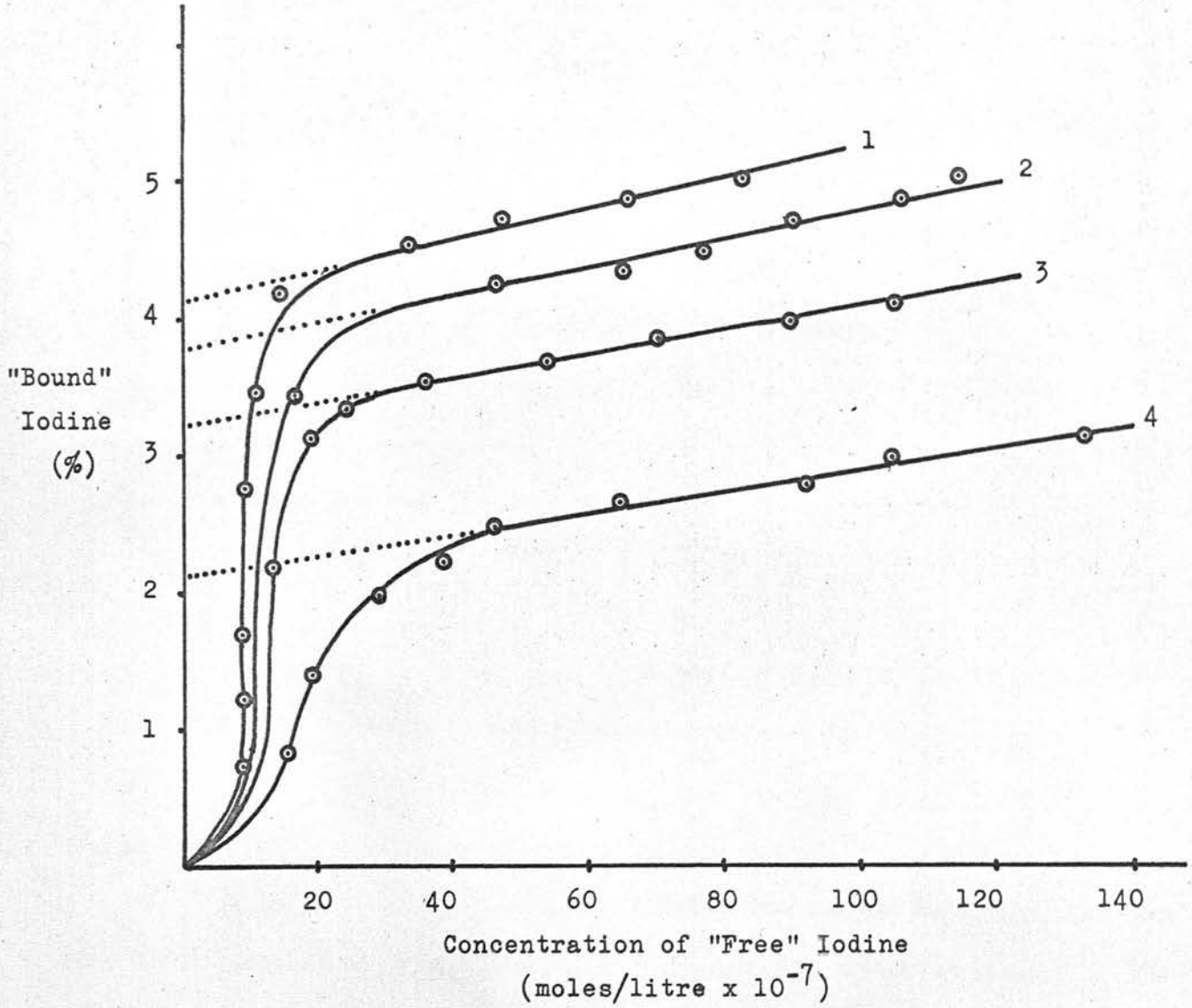
D.P. of chloral hydrate - nitrogen treated amylose = 232

D.P. of chloral hydrate - air treated amylose = 210

In view of the above results it was concluded that chloral hydrate does not cause extensive degradation of amylose. It seemed possible that the results of Anderson and King (1961) were due to small quantities of chloral hydrate interfering with their potentiometric iodine titrations. The effect of chloral hydrate on potentiometric iodine titrations was therefore investigated.

Starch (477·6 mg.) from potatoes (var. Duke of Kent) was dissolved in 0·5N-KOH (100 ml.) and neutralised with approximately 0·5N-HCl (105·5 ml.). Portions (20 ml.) were taken and 0·5N-

EFFECT OF CHLORAL HYDRATE ON
POTENTIOMETRIC IODINE TITRATIONS



1. No chloral hydrate.
2. 1.0g. chloral hydrate.
3. 3.0g. chloral hydrate.
4. 6.6g. chloral hydrate.

FIGURE 4

potassium iodide (10 ml.) added, followed by varying amounts of chloral hydrate solution and the volume made up to 100 ml. with distilled water. Potentiometric iodine titrations were carried out as described in the General Methods Section. The results are shown graphically in figure 4 and indicate that more than 0.5 g. of chloral hydrate per 40 mg. starch was necessary before interference occurred.

Chloral Hydrate Extraction

The cell suspension was diluted with methanol and treated with ultrasonic vibrations for 5 min. at 2°. The mixture was centrifuged and the residue exhaustively defatted with methanol and dried under vacuum. The dried cell material (1·70 g.) was added to a solution containing chloral hydrate (16·5 g.) in water (50 ml.), which had been heated to 85° in an atmosphere of nitrogen. The mixture was stirred vigorously under nitrogen at 80-85° for 1 hr., centrifuged, and the supernatant solution poured into acetone (800 ml.). The residue was extracted a further three times with chloral hydrate and the supernatant solutions poured into the same acetone solution. The precipitate, which formed, was centrifuged down, washed with acetone (x6) followed by ether (x4) and dried under vacuum.

Yield of chloral hydrate extracted material = 0·12 g.

Weight of residual material = 1·20 g.

Examination of chloral hydrate extracted material.

The material was almost pure white in colour, gave a red-purple stain with iodine and dissolved completely in boiling water. On total acid hydrolysis followed by neutralisation and examination by paper chromatography using 10:4:3 as solvent and aniline oxalate spray reagent, glucose (++++), galactose (+), mannose (±) and xylose (±) were detected. On quantitative acid hydrolysis followed by Somogyi estimation as glucose, the poly-glucose content was 18·2%. The material had a "blue value" of 0·12, λmax 510mμ, α-amylolysis limit about 75% and a β-amylolysis limit (at pH 4·6) of about 60%.

These preliminary results indicate the probable presence of an amylopectin-type polysaccharide.

Extraction using dimethyl sulphoxide.

The cell suspension was allowed to warm up to room temperature, centrifuged and the supernatant solution together with one methanol washing evaporated down and weighed. The solid material was washed three times with ice-cold water and then suspended in water. The cell suspension was cooled to 2°, disrupted with ultrasonic vibrations for seven minutes and centrifuged. The cell debris was washed with water followed by alcohol (x2) and ether (x6) and dried under vacuum. The washings were combined with previous washings.

Weight of dried cell material = 1·01 g.

Weight of chlorophyll, fat etc. = 1·10 g.

EXTRACTION WITH DIMETHYL SULPHOXIDE

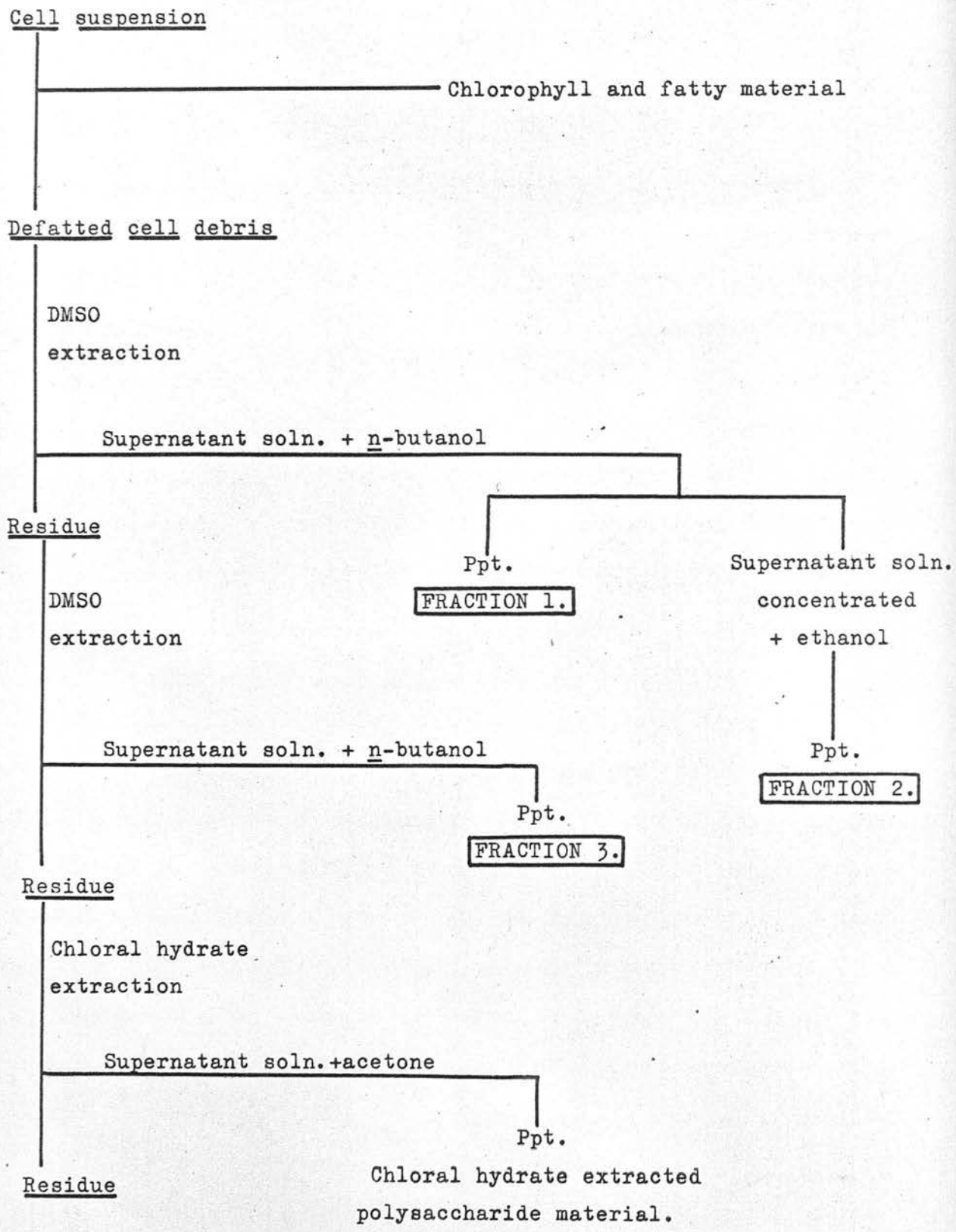


FIGURE 5

The dried cell material was suspended in dimethyl sulphoxide (100 ml.) and the mixture stirred in an atmosphere of nitrogen at room temperature for 6 days. The dimethyl sulphoxide suspension was centrifuged at 4,000 r.p.m. for 15 min. and the supernatant solution heated to 65° under nitrogen, with n-butanol (100 ml.). On cooling slowly to room temperature and centrifuging, a white precipitate was collected and dried. FRACTION I (80 mg.).

The supernatant solution from the butanol precipitation was evaporated to about 5 ml. and ethanol (4 ml.) added. A further precipitate was centrifuged down and dried. FRACTION 2 (85 mg.).

The residue from the above extraction was re-extracted in the same manner except that n-butanol (300 ml.) was added to the dimethyl sulphoxide extract. This precipitated all the polysaccharide in the extract, since a further addition of ethanol to the concentrated supernatant solution produced no precipitate. FRACTION 3 (12 mg.).

The residue was extracted with chloral hydrate solution (x3) under the conditions described on page 28 . The precipitate formed on addition of the chloral hydrate extracts to acetone weighed only 15 mg., had a polyglucose content of 17% and on total acid hydrolysis gave glucose (+++), galactose (+), mannose (±) and xylose (±). In view of this, it was not analysed further.

Allowing for the polyglucose content of each of the fractions, the yield of polysaccharide corresponded to 7% of the weight of dried cells.

Examination of Fraction 1.

The white material was soluble in warm water, producing a slightly opalescent solution and gave a red-purple stain with iodine. On incubation with salivary α-amylase, spots corresponding to maltose (+++) and glucose (++) were detected by paper chromatography, and the remaining solution gave no stain with iodine. With Wallerstein β-amylase at pH 4·6, maltose (+++) and glucose (±) were produced and the remaining solution gave a port-wine colour with iodine.

On partial acid hydrolysis, using 0·33N-H_2SO_4 for 45 min. at 100° followed by neutralisation with barium carbonate, five spots were readily detected on paper chromatography using 10:4:3 as solvent and silver nitrate spray reagent.

Spot	R_G	Standard	R_G
(a)	1·00	Glucose	1·00
(b)	0·69	Maltose	0·69
(c)	0·48	Maltotriose	0·48
(d)	0·32		
(e)	0·22		

The graph of -log R_G against the probable DP gave a straight line, indicating that the sugars formed a homologous series.

On total acid hydrolysis followed by neutralisation only glucose was detected and when the hydrolysate was incubated with a glucose oxidase preparation (5 mg. of a Takamine DeO enzyme preparation in 0·25 ml. of McIlvaine buffer pH 7·0) a single spot corresponding to gluconic acid was produced.

Polyglucose Content and β-Amylolysis Limit.

Fraction 1 (10·6 mg.) was weighed out accurately, dissolved in 2N-NaOH (2 ml.), neutralised with 2N-H_2SO_4, β-amylase (5 mg. in acetate buffer of pH 4·6) added and the solution diluted to 10 ml. with distilled water. Portions (1 ml.) were taken for acid hydrolysis followed by Somogyi estimation as glucose. Portions (3 ml.) were treated directly with Somogyi reagent for estimation of maltose. Glucose (0·989 mg./ml.) was produced, corresponding to a polyglucose content of 84·0% and maltose (0·559 mg./ml.) corresponding to a β-amylolysis limit of 59·4%.

Examination of Fraction 2.

The results of preliminary experiments on Fraction 2 were identical to those described for Fraction 1.

Polyglucose Content. Fraction 2 (10·5 mg.) was dissolved, diluted to 10 ml. and 1 ml. portions taken for acid hydrolysis and Somogyi estimation as described for Fraction 1. Glucose (0·696 mg./ml.) corresponding to a polyglucose content of 60% was found.

λmax of Iodine Complex. To 1 mg. of polysaccharide (1·59 ml.) was added 6N-HCl (0·03 ml.), water (5 ml.) and 1 ml. of a solution containing 0·2% iodine and 2% potassium iodide. The solution was diluted to 10 ml. with distilled water and the absorbance measured at various wavelengths against the corresponding blank.

$$\lambda max = 535 \text{ m}\mu.$$

Blue Value. 1 ml. of the iodine-polysaccharide solution used for

λmax determination was diluted to 10 ml. and the corresponding blank likewise diluted. The absorbance was measured at 680 mμ using 2 cm. cells and the answer doubled to give the "blue value."

"Blue value" of fraction 2 = 0·104.

Potentiometric Iodine Titration. Semi-micro potentiometric iodine titration was carried out as described in the experimental methods section. The polysaccharide solution used for polyglucose determination (4·5 ml.) was used. The "iodine affinity" was 0·60%.

Purification of Fraction 2. Fraction 2 (72 mg.) was dissolved in water and dialysed against 4 changes of distilled water. The dialysate was concentrated and contained no polysaccharide material. The mixture remaining in the dialysis bag consisted of a slightly opaque solution and a brown precipitate. The brown material was centrifuged down and washed with cold water. The solution and the washings were evaporated to about 30 ml. and the polysaccharide precipitated by addition of ethanol (120 ml.). After drying, the material had the following properties:-

Polyglucose content	86%
λ max of iodine complex	540 mμ
"Blue value"	0·102
"Iodine affinity"	0·38%.

Final Purification.

The material remaining from Fractions 1, 2 and 3 was combined (100 mg.) and dissolved, under nitrogen, in dimethyl sulphoxide

(10 ml.). The mixture was centrifuged at 18,000 r.p.m. for 15 min. yielding a brown precipitate, which was rejected, (cf. Be Miller and Whistler, 1962). The supernatant solution was treated with ethanol (3 vol.) and the precipitate washed thoroughly with ethanol and ether to remove dimethyl sulphoxide and dried. The white powder was dissolved in water (20 ml.) and precipitated with ethanol (80 ml.). The ethanol precipitation was repeated four times and the material then dialysed against four changes of distilled water. The material remaining in the dialysis bag was centrifuged and ethanol added to the supernatant solution. The precipitate was dissolved in 0·1M-NaCl (45 ml.) and shaken with toluene (7 ml.). The material, which collected at the interface, was rejected and the process repeated three times. The polysaccharide was recovered from the aqueous layer by ethanol precipitation and dried in the usual manner.

FRACTION 4 (50 mg.).

Examination of Fraction 4

Fraction 4 (8·1 mg.) was dissolved in water and diluted to 10 ml. Portions (1 ml.) were taken for acid hydrolysis and Somogyi estimation. The yield of glucose was 0·852mg./ml. corresponding to a polyglucose content of 94·7%. The specific rotation was measured in a 1 decimeter tube.

$$[\alpha]_D^{18} = +208^\circ \ (c = 0{\cdot}08, H_2O).$$

Methylation of Fraction 4.

The first stage of the methylation was carried out by the method of Kuhn and Trischmann (1963), as modified by Dr. D. A. Rees (personal communication). Fraction 4 (40 mg.) was dissolved in dimethyl sulphoxide (2·5 ml.), cooled to 0° and barium hydroxide octahydrate (2·5 g.) added. The mixture was stirred vigorously, with cooling, for 0·5 hr. to produce a thick white slurry. Dimethyl sulphate (0·45 ml.) was added and subsequent additions (0·45 ml. each) made 1, 1·5 and 2 hr. after the first addition, to make the total volume of dimethyl sulphate added 1·8 ml. 30 Min. after the last addition, the ice-bath was removed and the mixture allowed to warm up to room temperature. After 1 hr. the flask became warm and the white slurry changed to an almost clear oil, which was stirred at room temperature for 3 days under sealed conditions.

0·88-Ammonia (1 ml.) was added and stirring continued for 1 hr. The oil was extracted with warm chloroform (50-60 ml.) and centrifuged. Three layers separated; the top two were kept, while the bottom layer was re-extracted with hot chloroform (30 ml. x 3). The bottom oily layer was rejected and the top two layers (chloroform and gel) washed successively with water (50 ml. x 3), 2% EDTA solution of pH8 (50 ml. x 2) and finally with water (50 ml. x 3). At this stage, centrifugation produced two layers; the aqueous layer was rejected and the chloroform layer concentrated to a volume of about 5 ml., centrifuged, the residue (traces of inorganic matter) rejected, and the chloroform

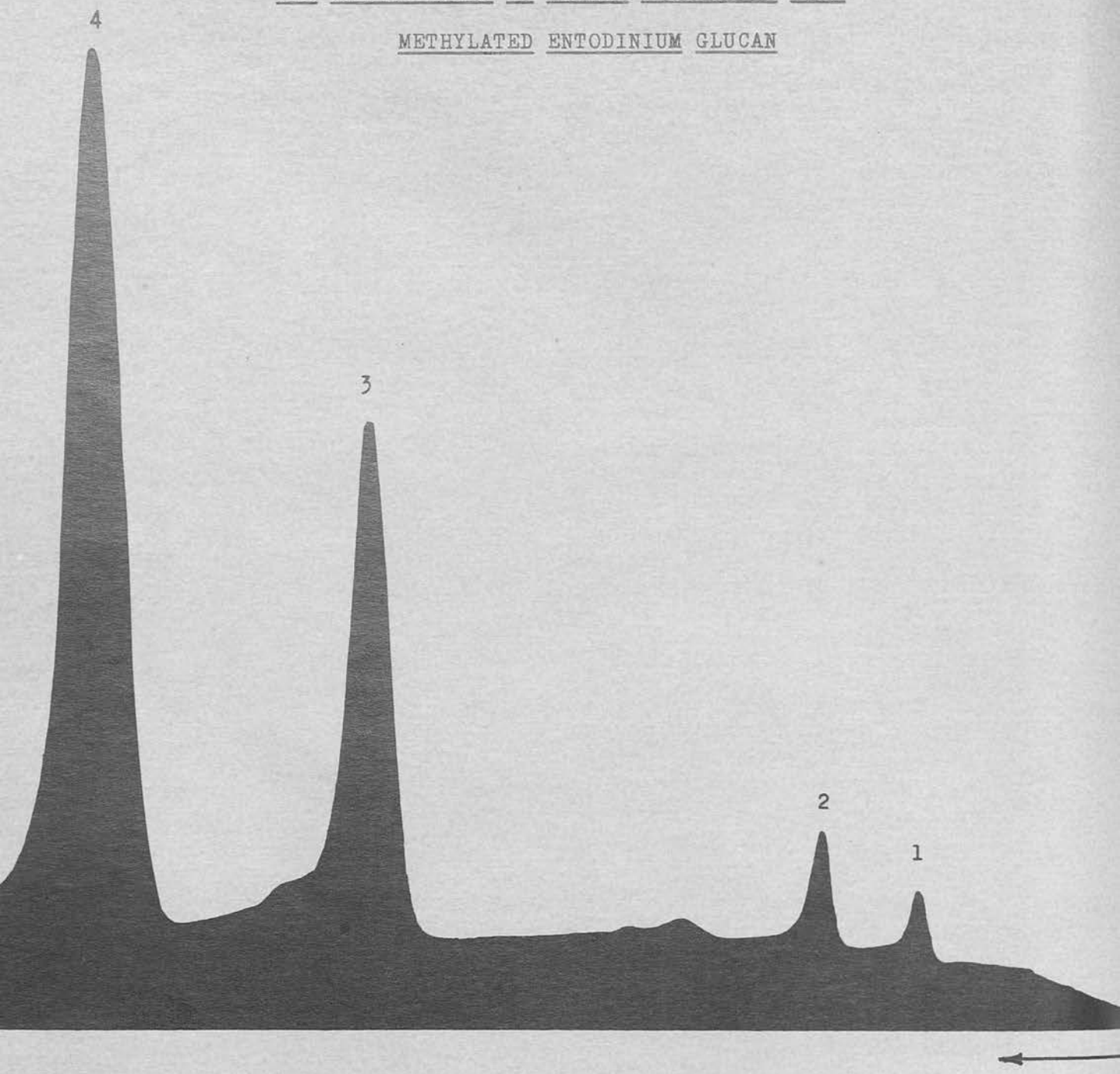
solution taken to dryness.

The second stage of the methylation was carried out by the method of Kuhn, Trischman and Löw (1955). The partly methylated product was dissolved in dimethyl formamide (2 ml.) followed by the addition of methyl iodide (2 ml.) and silver oxide (1 g.). The mixture was stirred overnight in a sealed container at room temperature and a further addition of methyl iodide (2 ml.) and silver oxide (1 g.) made. After stirring in the dark for 24 hr., the mixture was filtered and the silver oxide washed with warm chloroform. The solution and washings were evaporated to dryness (high vacuum was required to remove the dimethyl formamide).

The product was examined at this stage by paper chromatography of the hydrolysate. A portion of the product (ca. 2 mg.) was heated at 100° with 90% formic acid (0·5 ml.) for 1 hr. and the formic acid removed by successive evaporations with water. The hydrolysis was completed by heating the product at 100° with 2N-H_2SO_4 (1 ml.) for 2 hr., followed by neutralisation with barium carbonate. On paper chromatography using methyl ethyl ketone - water - ammonia (200:17:1, by vol.) as solvent and spraying with aniline oxalate, spots corresponding to authentic samples of 2,3,6-tri-O-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-D-glucose were detected. The trimethyl sugar gave a brown colour with aniline oxalate and ran slower than the 2,4,6-isomer, which gave a red colour.

Since two dimethylglucoses and traces of undermethylated products were also present, the second (silver oxide - methyl

GAS CHROMATOGRAM OF METHYL GLYCOSIDES FROM METHYLATED ENTODINIUM GLUCAN



1. Methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside.
2. Methyl 2,3,4,6-tetra-O-methyl-α-D-glucoside.
3. Methyl 2,3,6-tri-O-methyl-β-D-glucoside.
4. Methyl 2,3,6-tri-O-methyl-α-D-glucoside.

FIGURE 6

iodide) stage of the methylation was repeated. After this treatment, 33 mg. of methylated product were obtained, which, on examination by hydrolysis followed by paper chromatography, appeared to be well methylated.

The methylated glucan (ca. 2 mg.) was heated at 100° for 12 hr. with a 3% solution of methanolic hydrogen chloride (1 ml.) in a sealed tube. The hydrogen chloride was removed by successive evaporations with anhydrous methanol and the product examined by gas-liquid chromatography, (performed by Dr. G. O. Aspinall). The presence of 2,3,4,6-tetra-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-glucose was confirmed by the relative retention times of the methyl glycosides on a polyphenyl ether [m-bis-(m-phenoxy-phenoxy)-benzene] column and on a butan-1,4-diol succinate polyester column. The results from the latter column are indicated in Fig.6.

Determination of the Ratio of Methylated Sugars.

The product (20 mg.) was hydrolysed successively with formic acid and sulphuric acid as previously described. The methylated sugars in the neutralised hydrolysate were estimated by method 5 in the experimental methods section.

The molar ratios in three experiments were:-

	Dimethyl	Trimethyl	Tetramethyl
(a)	1·30	17·90	1·00
(b)	1·23	14·67	1·00
(c)	1·40	18·60	1·00

The average of these results gave a chain length of 19·4.

Extraction of Second Batch of Entodinium Cells.

The cell material was treated as previously described and two dimethyl sulphoxide extractions carried out. The entire polysaccharide material from the two extractions was combined and extracted with boiling water (100 ml. x1, 50 ml. x4) under nitrogen. The residue was grey in colour, gave no stain with iodine and was therefore rejected. Ethanol (4 vol.) was added to the supernatant solution and the precipitated material dissolved in water (30 ml.) and dialysed against four changes of distilled water. The brown material, which precipitated spontaneously, was removed and ethanol (4 vol.) added to the solution. The polysaccharide was dried, in the usual manner, before dissolving in warm dimethyl sulphoxide (10 ml.) under nitrogen. The solution was centrifuged at 18,000 r.p.m. for 10 min. and the brown precipitate rejected. The supernatant solution was treated with ethanol (3 vol.) and the polysaccharide dried. This process was repeated three times using dimethyl sulphoxide (7 ml.) and, on the final run, the polysaccharide dissolved readily in cold dimethyl sulphoxide giving a clear colourless solution (cf. BeMiller and Whistler, 1962). The polysaccharide was then precipitated three times from water using ethanol (4 vol.) and dissolved in 0·1M-NaCl (25 ml.). Toluene (5 ml.) was added and the mixture shaken vigorously for 24 hr. The toluene layer and the gel (centre layer) were removed and the process repeated four times. The saline solution was treated with ethanol (4 vol.) and a crystal of ammonium acetate (to aid

precipitation). The polysaccharide was washed successively with 75% ethanol (to remove traces of sodium chloride), ethanol, ether and dried.

To ensure that the extraction had been efficient, the residue from the two dimethyl sulphoxide extractions was extracted with chloral hydrate. The extraction procedure and results were identical to those described on page 30 for the first extraction.

Examination of the Highly Purified Polysaccharide.

Polyglucose Content. The polysaccharide (9·4 mg.) was dissolved in warm water and diluted to 10 ml. Portions (1 ml.) were taken for acid hydrolysis followed by Somogyi estimation of glucose. Glucose (1·007 mg./ml.) was produced corresponding to a polyglucose content of 96·4%.

Blue Value and λ max. Exactly 1 mg. of polysaccharide (1·102 ml.) from the above solution was delivered into a 10 ml. volumetric flask and 2N-HCl (0·05 ml.), water (5 ml.) and 1 ml. of a solution containing 0·2% iodine and 2% potassium iodide added. The solution was diluted to 10 ml. with distilled water (solution A). A blank was prepared containing 2N-HCl (0·05 ml.) and iodine solution (1 ml.) in a total volume of 10 ml. (solution B). Portions of solutions A and B were each diluted by a factor of 2 for λ max determination and by a factor of 10 for "blue value" determinations.

λ max = 540 mμ

"Blue value" = 0·102

POTENTIOMETRIC IODINE TITRATIONS OF SOME α-GLUCANS

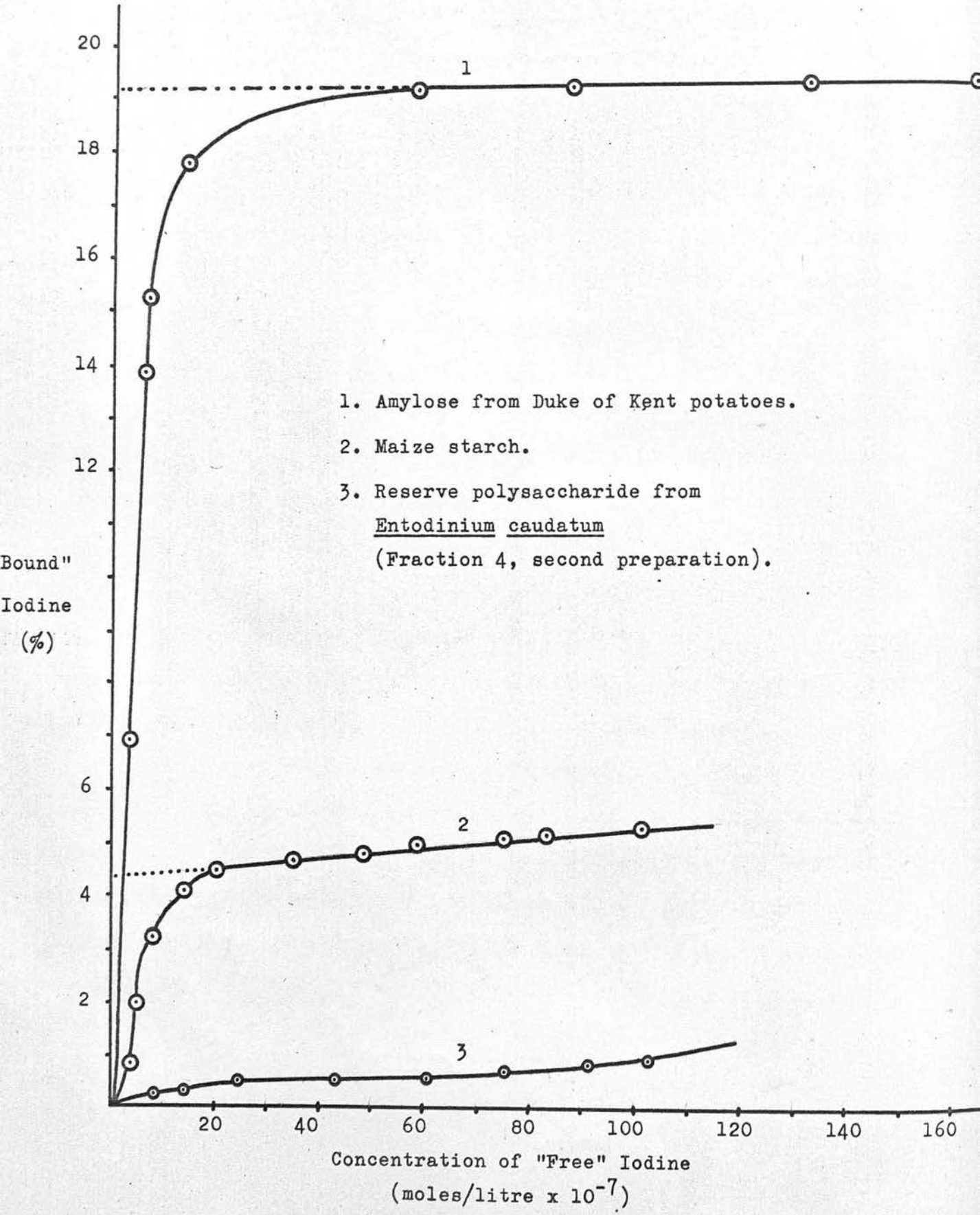


FIGURE 7

Potentiometric Iodine Titration. To the polysaccharide solution, used for polyglucose content determination, (4·5 ml.) was added potassium chloride (0·0186 g.) and 0·5N-potassium iodide (0·5 ml.). The titration was carried out as described in the experimental methods section using 0·000972 N-iodine solution.

ml.Iodine	mVs	mV_B	"Free" Iodine moles/litre $x10^{-7}$	"Bound" Iodine moles/litre $x10^{-7}$	%
0·10	228	259	8·0	87	0·255
0·15	234	263	14·1	127	0·377
0·20	241	266	25·4	161	0·483
0·30	251	270	60·0	214	0·654
0·35	254	272	75·5	242	0·745
0·40	256	273	92·4	267	0·830
0·45	258	275	103·0	298	0·935

These results gave an "iodine affinity" of 0·30%, and are compared graphically with a potato amylose and maize starch in figure 7.

β-Amylolysis Limit and Chain Length by α-Amylolysis of the β-Limit Dextrin.

Amylopectin (20-25 mg.) was dissolved by warming in 8 ml. of distilled water and cooled to 35°. To this was added β-amylase (5-6 mg.) in acetate buffer of pH 4·6 (10 ml.) (i.e. 25 units of enzyme per mg. of polysaccharide). All volumes were measured at 35°. Portions (0·5 ml.) were removed for acid hydrolysis and Somogyi estimation of the glucose liberated. After 24 hr. a portion (5 ml.) of the digest was diluted to exactly 11 ml.

Portions (5 ml.) of this solution were taken for Somogyi estimation of the maltose liberated by the action of the β-amylase.

The remainder of the original solution was heated at 100° for 15 min. to denature the β-amylase and then cooled to 35°. A portion (10 ml.) was neutralised to pH 6-8 using sodium hydroxide solution and carefully washed into a 25 ml. volumetric flask. Phosphate-citrate buffer (McIlvaine, 1921) of pH 7·0 (5 ml.) and 1·25 ml. of a solution containing 6·5 mg. of α-amylase preparation in 1·0% sodium chloride were added and the solution diluted to 25 ml. Portions (5 ml.) were taken for total maltose determinations.

The method described above is essentially that of Manners and Wright (1962), but the sample of α-amylase was the same as that used by Kjölberg, Manners and Wright (1963); the appropriate calibration equation is therefore taken from this latter paper.

The β-amylolysis limit of the amylopectin and the α-amylolysis limit of the β-limit dextrin are found by experiment and from the equation

$$P_{1,6} = 23{\cdot}4 - 0{\cdot}20P_M = \frac{100}{CL_1}$$

Where $P_{1,6}$ = percentage $1 \rightarrow 6$ links.

P_M = apparent percentage conversion to maltose by α-amylase.

CL_1 = chain length of β-limit dextrin

CL_2 = chain length of amylopectin.

the chain length of the β-limit dextrin can be calculated.

Now $$CL_2 = \frac{CL_1 \times 100}{100 - \beta\text{-limit}}$$

and hence the chain length of the amylopectin can be calculated.

To check this procedure, chain lengths of potato, oat and maize amylopectins were measured by the above procedure and by periodate oxidation (for method see page 55).

Amylopectin	Chain length by periodate	Chain length by enzymic assay
Potato	23.0	22.9
Oat	19.6	20.1
Maize	22.5	21.4

Results from Entodinium Amylopectin:-

β-Amylolysis limit = 58.4%

α-Amylolysis limit of β-limit dextrin = 54.6%

Average chain length of β-limit dextrin = 8

Average chain length of amylopectin = 19.2

Hence, average internal chain length = 4-5

Average external chain length = 13-14.

Discussion.

The present experiments were carried out on suspensions of *Entodinium caudatum* cells isolated by Dr. J. M. Eadie (Rowett Research Institute, Aberdeen). The rumen fluid was taken at a time when the organisms were free from plant starch and contained only storage polysaccharide. Treatment with chloramphenicol killed the bacteria and mannose was used to kill the *Dasytricha ruminantium*. The cells were washed and stored at -20° until required.

The method of Clarke and Stone (1960) proved unsuccessful in the extraction of this storage polysaccharide. The reasons for this are possibly because the storage polysaccharide is present only in small amounts and that it is fairly soluble in urea solution and perhaps even in cold water. This would cause a distribution of the polysaccharide over fractions containing protein and other impurities. It is notable that the method of Clarke and Stone has previously been applied successfully to the extraction of protozoal polysaccharides which are (a) present in fairly high proportions and (b) very insoluble in aqueous solutions (see, for example Cunningham, Manners and Ryley, 1961; and Clarke and Stone, 1960). The failure of the Clarke and Stone method was not due to amylase activity in the trypsin.

Before using chloral hydrate solution as a means of extraction, it was tested for possible degradative effect. Aqueous chloral hydrate has been used in the past for extraction of α-glucans (see

for example Bourne, Stacey and Wilkinson, 1950; Forsyth and Hirst, 1953) but more recently it has been reported to cause degradation (Anderson and King, 1961). Experiments carried out in the present investigation showed that an amylose of DP 250 was degraded only to a DP of 232 when treated with 33% aqueous chloral hydrate at 80° for 1 hr. under anaerobic conditions, and to a DP of 210 under aerobic conditions. It is therefore concluded that chloral hydrate solution does not cause extensive degradation of α-(1→4)-linked chains of glucose units. It seemed possible that the results of Anderson and King were caused by incomplete removal of chloral hydrate from their sample; however, the present results indicate that very large amounts of chloral hydrate (more than 0·5 g. per 0·04 g. starch) are necessary before interference occurs with potentiometric iodine titrations. Such quantities would certainly not be encountered.

Extraction of the Entodinium caudatum suspension with 33% aqueous chloral hydrate yielded an iodophilic polysaccharide with a polyglucose content of 18%. The results of iodine staining and enzymic experiments indicated that it was possibly an amylopectin ("blue value," 0·12; λ max, 510mμ; β-amylolysis limit, 60%; α-amylolysis limit, 75%). The significance of the small amounts of galactose, mannose and xylose present in the hydrolysate of the chloral hydrate extract is not known.

The most efficient and selective method of extraction involved the use of dimethyl sulphoxide. This solvent has recently been used in studies on α-glucans by several workers (Leach and Schoch,

1962; Killion and Foster, 1960; BeMiller and Whistler, 1962). In an initial extraction, a cell suspension was disrupted by ultrasonic vibrations and the defatted cell debris extracted with dimethyl sulphoxide for 6 days. On addition of n-butanol (1 vol.) to the supernatant solution, a precipitate was formed (Fraction 1) which had a polyglucose content of 84%, and was partly degraded by α and β-amylases yielding maltose as the main product in each case. On total acid hydrolysis fraction 1 gave only glucose, the presence of which was verified by its complete conversion to gluconic acid when treated with a glucose oxidase preparation; and on partial acid hydrolysis a homologous series of oligosaccharides was produced with R_G values identical to the maltosaccharides. Fraction 1 had a β-amylolysis limit of 59%.

The supernatant solution from the first dimethyl sulphoxide extraction was concentrated and treated with ethanol yielding a second precipitate (fraction 2). Fraction 2 resembled fraction 1 in being an iodophilic polymer of D-glucose; it had a polyglucose content of 60%; "blue value," 0·104; λmax, 535mμ; and an "iodine affinity" of 0·60%. It was found possible to purify fraction 2, by dialysis and ethanolic precipitation, to a polyglucose content of 86% and the iodine staining properties were only slightly modified.

The residue from the first dimethyl sulphoxide extraction was re-extracted with this solvent and all the iodine staining material in the extract precipitated with butanol (3 vol.) (fraction 3). The residue was extracted with chloral hydrate

yielding only a very small amount of impure polysaccharide material which possessed glucose, galactose, mannose and xylose as constituent sugars; thus emphasising the selectivity of the dimethyl sulphoxide extraction. The remaining portions of fractions 1,2 and fraction 3 were combined and purified by high speed centrifugation in dimethyl sulphoxide, dialysis, precipitation with ethanol and toluene-sodium chloride solution treatments. The product (fraction 4) had a polyglucose content of 95% and a specific rotation $[\alpha]_D^{18} = +208$ ($c = 0{\cdot}08$, H_2O). Fraction 4 was methylated with barium hydroxide and dimethyl sulphate in dimethyl sulphoxide by a modification of the method of Kuhn and Trischmann (1963), followed by two treatments with silver oxide and methyl iodide in dimethyl formamide (Kuhn, Trischmann and Löw, 1955). The product (obtained in 65% yield) on acid hydrolysis and examination by paper chromatography yielded spots corresponding to authentic samples of 2,3,4,6-tetra-O-methyl-D-glucose; 2,3,6-tri-O-methyl-D-glucose; and dimethylated products. The 2,3,6-tri-O-methyl-D-glucose was quite different from an authentic sample of the 2,4,6-isomer both in its colour reaction with aniline oxalate and in its chromatographic mobility. Confirmation of the presence of 2,3,4,6-tetra-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-glucose was obtained by methanolysis of a sample of the product and examination of the mixed glycosides using a Pye Argon Chromatograph, using two different columns (Aspinall, 1963).

The molar ratio of methylated sugars, estimated by the method of Schaefer and Van Cleve (1956), was tetramethyl-glucose: trimethyl-

glucose:dimethyl-glucose = 1·00:17·06:1·31, corresponding to an average chain length of 19·4.

A second preparation of fraction 4 was made using slight modifications in the methods of purification. The yield was 40 mg. and the polyglucose content 96-97%. The results of iodine staining experiments were consistent with the polysaccharide being an amylopectin ("blue value" 0·102, λmax 540 mμ, "iodine affinity" 0·30). The chain length was found by the small scale enzymic technique of Manners and Wright (1962). Since the direct determination of α-amylolysis limit on amylopectins does not give an accurate estimate of chain length the β-limit dextrin was prepared and its chain length determined with α-amylase. Although the determination of chain lengths of glycogens by α-amylolysis is well established, the above procedure for amylopectins has only been carried out on a few samples. In view of this, oat, maize and potato amylopectins were prepared and their chain lengths determined by periodate oxidation under the conditions of Perlin (1954) and by α-amylolysis of the corresponding β-limit dextrins. The values obtained by the two methods were in good agreement. The value for the chain length of Entodinium caudatum amylopectin was 19 (cf. 19-20 obtained by methylation studies on the first preparation of fraction 4. Using the chain length of 19 in conjunction with the β-amylolysis limit of 58% the average exterior chain length (E.C.L.) is 13-14 and the average interior chain length (I.C.L.) is 4-5. The properties of Entodinium caudatum amylopectin are compared with those of other amylopectins and a glycogen in Table 1.

TABLE I PROPERTIES OF SOME AMYLOPECTINS

Source of amylopectin	Blue value	λmax (mμ)	Iodine Affinity %	β-Limit %	C.L.	E.C.L.	I.C.L.
Entodinium caudatum	0·10	540	0·30	58	19	13-14	4-5
Potato	0·14	550	0·15	57	23	15-16	5-6
Oat	0·07	545	0·24	55	20	13-14	5-6
Maize	0·15	555	0·18	58	23	16	6
Polytoma uvella	0·12	545	-	58	23-24	16	7
Glycogen * (Rabbit Liver XVII)		450	-	48	14	9	3

* Results from Kjölberg, Manners and Wright (1963).

It is therefore concluded that the reserve polysaccharide from Entodinium caudatum is an amylopectin. This puts Entodinium caudatum in the same category as Cycloposthium, a ciliated protozoan found in the colon and caecum of the horse (Forsyth, Hirst and Oxford, 1953) and holotrich ciliates from the rumen of sheep (Forsyth and Hirst, 1953).

SECTION (D)

STUDIES ON THE RESERVE POLYSACCHARIDE FROM POLYTOMA UVELLA

Introduction

Polytoma uvella is a flagellated protozoan found in sewage and in decaying protein solutions. Although the organism contains no chlorophyll, it has been shown to synthesise relatively large quantities of starch granules from fatty acids; sugars can not be utilised as a source of carbon (Pringsheim, 1937). This author assumed that the granules were composed of starch from (a) their blue coloration with iodine and (b) they were attacked by amylases to give reducing sugars.

Recently Dr. G. A. Mercer isolated the granules in a highly purified form, using the method of Clarke and Stone (1960). On examination of the whole starch Dr. Mercer obtained the following results:-

(i) On total acid hydrolysis only glucose was produced.

(ii) Blue value 0·244.

(iii) Iodine affinity 3·1%.

(iv) Specific rotation + 149° (c 0·9, 1N-NaOH).

(v) Average chain length 31.

(vi) β-Amylolysis limit 58%.

Attempts to fractionate the starch failed, apparently because of difficulties involved in dispersion of the granules. Thegranules

were insoluble in boiling water, even after pretreatment with liquid ammonia. An additional hazard was that the granules were so small that they passed through a G2 sinter glass filter; this made it difficult to see when they were completely in solution.

In the present investigation the fractionation experiments have been continued and the results of Dr. Mercer discussed in the light of new information on the component fractions of the starch.

EXPERIMENTAL

Fractionation Experiments (a) Conventional Method.

The starch isolated from Polytoma uvella by Dr. G. A. Mercer had proved resistant to fractionation. Before proceeding with this experiment, the starch was exhaustively defatted with methanol in a Soxhlet apparatus. The defatted starch (350 mg.) was made into a paste with methanol and introduced into boiling, oxygen-free, water (350 ml.) under nitrogen. Even after boiling and vigorous stirring for 2·5 hr. gelatinous particles were present. Since centrifuging at this stage would have exposed the solution to oxygen, it was cooled to 60°, thymol (0·4 g.) added and the mixture cooled to room temperature. The undissolved starch was readily removed by centrifuging for 2 min. at 1,500 r.p.m. (this speed would certainly not bring down the amylose-thymol complex, had any been formed). After drying, the undissolved starch weighed about 300 mg. The supernatant solution was left for 3 days at room temperature and on centrifuging at 16,000 r.p.m. a "slimy" precipitate was removed. The solution was saturated with thymol at 60° and set aside for a further 3 days and a small additional amount of thymol precipitate removed by high speed centrifugation and combined with the first. The precipitate was thoroughly washed with ethanol and dried (yield, about 15 mg.). The supernatant solution was concentrated, treated with ethanol (6 vol.) and dried to yield 15 mg. of white powder. The two fractions both had λmax in the range 590-610mμ. The above method therefore gives no fractionation of the starch.

(b) 5N-KOH, Butanol treatment.

The starch (0·5 g.) was made into a paste with warm methanol and 5N-KOH (50 ml.) added. The mixture was stirred under nitrogen for 4 hr. After this time the starch was not all in solution and it was heated to 40° for 5 min. and cooled. The solution was neutralised with sulphuric acid to pH 7 (pH-meter) and the potassium sulphate crystals produced, filtered off. The temperature was raised to 70°, under nitrogen and the solution saturated with n-butanol. After cooling slowly to room temperature, a precipitate was formed, which was removed by centrifuging at 5,000 r.p.m. for 30 min. The precipitate was dissolved in water and re-precipitated with n-butanol. The complex was washed thoroughly with ethanol and ether and dried (amylose I). The supernatant solution was extracted with ether to remove butanol, dialysed for 48 hr. against tap water and freeze-dried (amylopectin I).

Determination of "blue value," λmax of the amylose and amylopectin and the "iodine affinity" of the amylose were carried out as described in the experimental section. The values found were:

	Amylose I	Amylopectin I
"Blue value"	0·57	0·20
λmax	630 mμ	570 mμ
"Iodine affinity"	6·2%	-

(c) 2N-NaOH, Thymol Treatment.

The starch (0.5 g.) was heated at 40° for 2 hr. in 2N-NaOH (40 ml.) and for a further 0.5 hr at 60°. Throughout the experiment nitrogen was passed through the solution, which was then cooled, neutralised with sulphuric acid to pH 7 (pH-meter) and heated to 70°. Thymol (100 mg.) was added and the solution allowed to stand at room temperature for 3 days in an atmosphere of nitrogen. The amylose-thymol complex was removed by centrifuging at 16,000 r.p.m. and then reprecipitated from water with n-butanol three times, (amylose II). The supernatant solution from the thymol precipitation was dialysed against tap water for 48 hr. and freeze-dried, (amylopectin II).

Examination of Amylose II. Amylose II was examined by the techniques described in the experimental methods section. The results were as follows:-

"Blue value" = 0·60

λmax = 635 mμ

"Iodine affinity" = 7·1%

β-Amylolysis limit = 95%

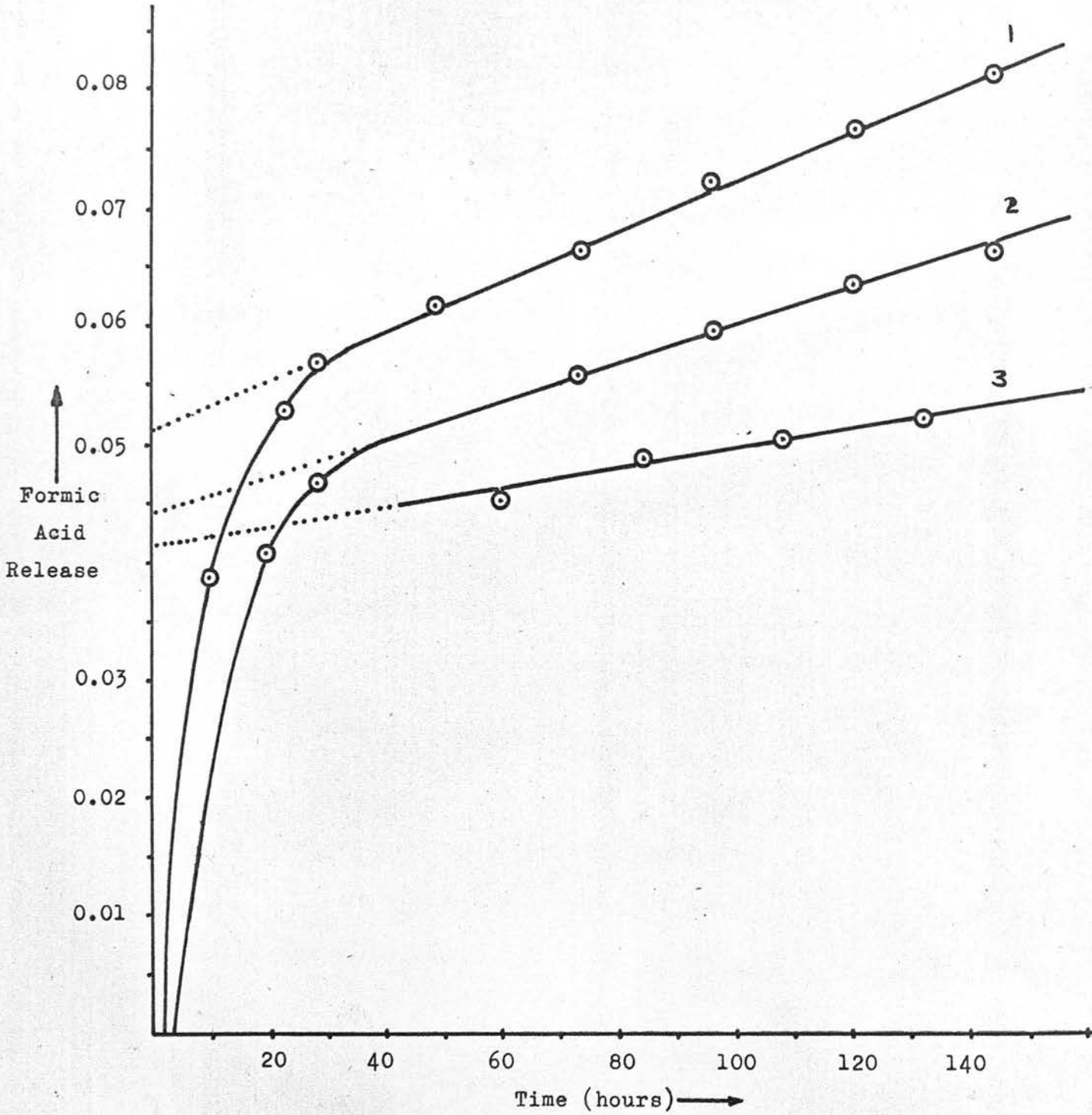
Examination of Amylopectin II. The results from amylopectin II were:-

"Blue value" = 0·12

λmax = 545 mμ

The average chain length of amylopectin II was determined by the method of Manners and Wright (1962), as described for Entodinium

PERIODATE OXIDATION OF SOME AMYLOPECTINS



1. Oat amylopectin.
2. Maize amylopectin.
3. Polytoma uvella amylopectin.

FIGURE 8

amylopectin p. 40.

β-Amylolysis limit = 58.4%

α-Amylolysis limit of β-dextrin = 65.2%

Hence, average chain length of β-dextrin = 9.9

Average chain length of amylopectin II = 23.3

Average exterior chain length = 16

Average interior chain length = 7

Determination of Chain Length by Periodate Oxidation.

An aqueous solution of amylopectin II (79·0 mg.) was adjusted to pH 5·8, 5% $NaIO_4$ (10 ml.) added and diluted to exactly 50 ml. with distilled water. The solution was kept in the dark at room temperature and samples(10 ml.) treated with ethylene glycol (4-5 drops) for 15 min. before titrating with 0·00323N-NaOH. The titration was carried out using a pH-meter and nitrogen was bubbled through the solution to prevent atmospheric carbon dioxide interfering. pH 5·8 was taken as the end-point.

Time (hr.)	Titre (ml.)	Formic acid release (mol.prop./glucose residue)	Apparent chain length (glucose residues)
60	1·35	0·0449	22·3
84	1·47	0·0488	20·5
108	1·51	0·0500	20·0
132	1·57	0·0521	19·2

From figure 8, facing page 55, the extrapolated value of the formic acid liberated was 0·0418 moles per anhydroglucose unit corresponding to an average chain length of 23·9. A comparison with parallel experiments on oat and maize amylopectins is also indicated in figure 8.

Discussion

The present investigation has been concerned with attempts to fractionate the starch from Polytoma uvella into amylose and amylopectin components. It was thought that the failure of Dr. G. A. Mercer's attempts to fractionate the polysaccharide might be due to fatty material adhering to the granules and preventing them from dissolving in boiling water. However, it was found that even after exhaustive defatting and treating with boiling water for 2·5 hr. the material did not dissolve. Fractionation by the conventional method was therefore not possible. Bourne, Stacey and Wilkinson (1950) found similar difficulties with the starch-type polysaccharide from Polytomella coeca.

In order to ascertain that the starch did, in fact, contain two components, a preliminary experiment was carried out in which the starch was dissolved in 5N-KOH. Even with this drastic treatment, it was necessary to heat the solution to 40° for a few minutes to obtain complete dissolution. The experiment was carried out with nitrogen bubbling continuously through the solution and, after neutralisation and saturating the solution with n-butanol, a precipitate was formed, which was purified by a further butanol precipitation, (amylose I). The supernatant solution was extracted with ether, dialysed and freeze-dried, (amylopectin I).

The iodine-staining properties of the two fractions indicated that fractionation into amylose and amylopectin-type components

had been achieved.

	Amylose I	Amylopectin I
"Blue value"	0·57	0·20
λmax	630 mμ	570 mμ

In a second alkaline dispersion experiment the starch was dissolved by treatment in 2N-NaOH for 2 hr. at 40°. After neutralisation, addition of thymol at 60° and leaving at room temperature for 3 days, a precipitate was formed, which was recrystallised three times from aqueous solution using n-butanol and the product dried, (amylose II). The supernatant solution was extracted with ether to remove thymol and then freeze-dried (amylopectin II).

Amylose II ("Blue value" 0·60, λmax 635 mμ) was shown to be an essentially linear polymer by its β-amylolysis limit of 95%. However, the "iodine affinity" of 7·1% was lower than most amyloses indicating that it may have degraded during the fractionation, despite the anaerobic conditions.

Amylopectin II had the properties of a typical plant amylopectin ("blue value" 0·12, λmax 545 mμ and β-amylolysis limit 58%). The average chain length was found both by the enzymic method of Manners and Wright (1962) and by measuring the formic acid released when the polysaccharide was treated with sodium metaperiodate. The results from each experiment indicated an average chain length of 23-24 glucose units. From this value and the β-amylolysis limit, the average exterior chain length is 16 and the average interior chain length is 7.

The reserve polysaccharide from Polytoma uvella is therefore thought to be a typical starch. The values found by Dr. G. A. Mercer for the "iodine affinity" (3·1%), specific rotation (+149°) and β-amylolysis limit (58%) are almost certainly anomalous, and are probably due to the polysaccharide being incompletely dissolved. The present experiments indicate that the extreme insolubility of the starch would make it difficult to obtain the true values for these properties; if the starch is treated drastically enough to obtain complete dissolution, then the amylose component may be severely degraded. The answer may lie in some type of pretreatment of the granules. Preliminary experiments with dimethyl sulphoxide pretreatment have so far proved unsuccessful, and G. A. Mercer has shown that liquid ammonia pretreatment does not aid dissolution.

The present experiments have, however, shown that the amylopectin component is not severely degraded under the conditions of fractionation used. The detailed properties of the amylopectin are compared with other amylopectins in Table 1, p. 48 , and the properties of the whole starch and its components compared with those of other starches in Table 2, p.59.

TABLE 2. Properties of some Starches.

Starch		"Blue value"	λmax (mμ)	"Iodine affinity" (%)	β-Limit (%)	C.L. (by periodate oxidation)	C.L. (by enzymic assay)
Polytoma uvella	*Whole starch	0·244	-	3·1	58	31	-
	Amylopectin	0·12	545	-	58	24	23
	Amylose	0·60	635	7·1	95	-	-
Oat	Whole starch	0·40	605	4·00	65-66	-	-
	Amylopectin	0·07	545	0·24	55	20	20
	Amylose	1·52	677	18·5	98	-	-
Potato	Whole Starch	0·42	595	3·92	65-66	-	-
	Amylopectin	0·14	555	0·15	57	23	23
	Amylose	1·50	675	19·2	99	-	-
Maize	Whole starch	0·43	605	4·40	67-68	-	-
	Amylopectin	0·15	555	0·18	59	23	21
	Amylose	1·10	660	16·2	93	-	-

* Results from G.A. Mercer (1962) Ph.D. Thesis, University of Edinburgh.

CHAPTER 2

β-GLUCANS FROM PROTOZOA

Introduction.

Since a comprehensive review of β-(1→3)-glucans has been published recently (Clarke and Stone, 1963) the following account deals mainly with those β-glucans which are related to the polysaccharides examined in the present studies and with β-glucans which have been reported since the publication of Clarke and Stone's review.

β-Glucans containing (1→3)-glucosidic linkages are widely distributed throughout the plant kingdom; they have been isolated from fungi, algae, lichens and from higher plants. They also occur in several protozoa which are closely related to the algae. The glucans may serve either as structural or storage material. If the amount of the glucan diminishes, when the organism is under nutritional stress, then it is generally assumed that the glucan is serving as reserve or storage polysaccharide.

The physical properties of β-(1→3)-glucans appear to be governed by two main factors: (a) the degree of polymerisation and (b) the inclusion of linkages other than the main β-(1→3)-type. Laminaribiose is readily soluble in water, but as the series is ascended the solubility decreases. The inclusion of β-(1→4)-linkages in the linear chain render the polymer soluble. For example, lichenin is a linear polymer of glucose units linked by β-(1→3) and β-(1→4)-glucosidic linkages in the ratio 70:30 respectively (Chanda, Hirst and Manners, 1957) and (Peat, Whelan and Roberts, 1957) and, although it has a high degree of polymerisation, it is soluble in water. In contrast to this, callose

from the grape vine consists almost entirely of β-(1→3)-linked glucose units (Aspinall and Kessler, 1957) but is insoluble in dilute acids and in dilute alkali.

Laminarin.

Laminarin, which forms a reserve carbohydrate in various species of brown marine algae, exists in two forms which differ in solubility in cold water. A "soluble" form is present in Laminaria digitata whilst "insoluble" laminarin has been isolated from L.cloustoni. Peat, Whelan and Lawley (1955) showed, by partial acid hydrolysis, that mannitol was a constituent residue of laminarin; confirmation of this, together with evidence for a low degree of branching, was deduced from periodate and methylation studies (Anderson, Hirst, Manners and Ross, 1958). About half the molecules in laminarin are terminated by mannitol residues and separation of the mannitol-terminated chains from the glucose-terminated chains has been achieved by methods involving bromine oxidation and ion-exchange resin (Goldstein, Smith and Unrau, 1959). The isolation of ethylene glycol after periodate oxidation, boro-hydride reduction and hydrolysis indicates that the mannitol units are linked through C(1) (Annan, Hirst and Manners, 1962).

Leucosin.

"Leucosin" is characteristic of the Chrysophyceae and is a substance formed in large amounts by planktonic organisms in lakes and in sea. For many years leucosin has been generally regarded as a polysaccharide, although absolute chemical evidence was absent.

Under the microscope, the appearance of leucosin may resemble that of glycogen, but the former does not stain with iodine. Stosch (1951) demonstrated that in Synura (Chrysophyceae) and various diatoms, precipitation of leucosin occurred under the influence of "dehydrating" substances. This author considers that, in view of the derived mono- and disaccharides, leucosin is closely related to laminarin. In experiments on Ochromonas malhamensis, Pringsheim (1952) showed that by applying a moderate temperature (not high enough to denature the enzymes, but high enough to dismember the cells) enzymes were set free which hydrolysed the leucosin to reducing sugars. The structure of leucosin from Ochromonas malhamensis is discussed in chapter 2, section C.

Quillet (1955) found that leucosin from Hydrurus foetidus had a low specific rotation, did not stain with iodine and gave only glucose on total acid hydrolysis.

Leucosin, isolated from a mixture of diatoms, belonging to the Chrysophyceae, was examined by Beattie, Hirst and Percival (1961). The polysaccharide resembles laminarin in that it consists of about 20 glucose residues linked mainly by β-(1→3)-glucosidic linkages. Mannitol is, however, absent and these authors suggested, on the basis of methylation studies, that their sample of leucosin contains a small proportion of (1→6)-linkages and (1→6)-branch points.

Paramylon

A unique feature of the colourless and the green euglenoid flagellates is that most of them synthesise a characteristic reserve carbohydrate material known as paramylon (or paramylum). Several workers have shown that paramylon granules yield glucose on hydrolysis (Gottlieb, 1850; Habermann, 1874) and the former author has also shown that the granules resemble starch both in their general appearance and in their empirical formula. However, the paramylon granules do not stain with iodine and do not give any of the histochemical or cytological tests for carbohydrate material (details of these tests are summarised by Nath, Dutta and Dhillon, 1960). Most of these tests are based on iodine staining or on periodate oxidation and an explanation of the peculiar inertness of the paramylon granules towards these reagents was not put forward until Kreger and Meeuse (1952) suggested a similarity between the alkali-soluble β-glucan from yeast and paramylon granules from Euglena (mixed viridis and geniculata). The X-ray powder photographs of the two polysaccharides were identical and thus the presence of β-(1→3)-linkages in paramylon was inferred. Several years later, Clarke and Stone (1960) provided chemical evidence that the paramylon granules from Euglena gracilis were composed largely of glucose units linked by β-(1→3)-linkages. The granules were insoluble in water but dissolved in 2·5N-NaOH ($[\alpha]_D^{16}$ +28°) and, on partial acid hydrolysis, spots corresponding to the laminarisaccharides were detected by paper chromatography. The periodate uptake was only 0·02 mol.prop./

glucose residue. The paramylon granules from Peranema trichophorum were examined by Cunningham and Manners (1962) both by enzymic degradation and by periodate oxidation and the basic structure was shown to be the same as that of the paramylon granules from Euglena gracilis. In the present investigation work has been carried out on the paramylon from Peranema trichophorum and from Astasia ocellata (see chapter 2, sections D and E). The paramylon granules from Astasia longa (the colourless counterpart of Euglena gracilis) also consist largely of β-(1→3)-linked glucose units (Picciolo, 1963).

The biological aspects of paramylon synthesis in Khawkinea sp. have been examined (Nath, Dutta and Dhillon, 1960 and 1961). It has been shown that in non-growing cells of Euglena gracilis 80-90% of assimilated acetate^{14} C is found in the paramylon granules (Marzulla and Danforth, 1962). The presence of laminaribiose phosphorylase in extracts of Euglena gracilis has been demonstrated (Marechal and Goldemburg, 1963) and D.J.Manners and D.Taylor (unpublished work) have shown that cell-free extracts of Astasia ocellata also contain this enzyme;-

laminaribiose + inorganic phosphate $\rightleftharpoons$ α-glucosyl phosphate + glucose.

It is therefore possible that the synthesis of paramylon granules follows a similar mechanism to the synthesis of callose from Phytophthora cactorum (Eschrich, 1960). Recently, however, uridine 5-(D-glucosyl pyrophosphate) (UDPG) has been implicated in the synthesis of paramylon granules from Euglena gracilis (Marechal and Goldemburg, 1963a).

Fungal β-(1→3)-Glucans.

Partial acid hydrolysis studies have shown that the glucan from Poria (Pachyman) cocos consists of linear chains of glucose units linked by β-(1→3)-glucosidic linkages, Warsi and Whelan (1957). The polysaccharide (pachyman) therefore provides a useful source of the laminarisaccharides.

Recently, several other fungal glucans have been examined. Enzymic and periodate studies on the glucan from an unidentified Fungi imperfecti have shown that it has an average degree of polymerisation of 110 and consists of a linear backbone of about 85, β-(1→3)-linked glucose units with about 25 single glucose units attached to the main chain by β-(1→6)-glucosidic linkages (Johnson, Kirkwood, Misaki, Nelson, Scaletti and Smith, 1963). The basic structure of this glucan is shown in figure 13 facing p. 82. Other β-(1→3)-glucans with β-(1→6)-branch linkages have been isolated from Pullularia pullulans (Bouveng, Kiessling, Lindberg and McKay, 1963), Claviceps purpurea (Perlin and Taber, 1963) and Microsporum quinckeanum (Alfes and Bishop, 1963).

Section (B)

Experimental Methods

Drying of polysaccharide samples, evaporation of solutions, and estimation of reducing sugars (Somogyi, 1952) were carried out exactly as described in chapter 1, section (B).

Paper Chromatography.

Chromatograms were developed on Whatman No.1 paper using the following solvents:-

Solvent	Ratio (v/v/v)	Sugars separated	Development time (hr.)
Ethyl acetate/pyridine/water	10:4:3	monosaccharides	24
Butanol/pyridine/water	6:4:3	oligosaccharides	48
Methyl ethyl ketone/acetic acid/water (saturated with boric acid)	9:1:1	glucose & mannose	48-72
		glucose & mannitol	17
n-Butanol/ethanol/water (top layer)	4:1:5	methylated sugars	12
Methyl ethyl ketone/water/ ammonia	200:17:1	methylated sugars	3-4

These solvents are referred to by the ratio of the components, throughout the text. The silver nitrate and aniline oxalate spray reagents described on p.12 were used. Mannitol and sorbitol were detected after development in 9:1:1 by the periodate-permanganate spray reagent (Lemieux and Bauer, 1954).

Total Acid Hydrolysis.

(a) Polysaccharide samples from Ochromonas malhamensis (10 mg.)

were hydrolysed with 2N-H_2SO_4 (1 ml.) at 100° for 2 hr., followed by neutralisation with barium carbonate, for qualitative experiments. For quantitative experiments neutralisation was carried out with 2N-NaOH (phenolphthalein) and the solution made very slightly acid before estimation of glucose with Somogyi reagent.

(b) Paramylon granules from Peranema trichophorum and from Astasia ocellata were insoluble in 2N-H_2SO_4 at 100°. The granules (10 mg.) were hydrolysed with 90% formic acid (1 ml.) at 100° for 2 hr. 2N-H_2SO_4 (2 ml.) was added while the solution was still hot and heating, at 100°, continued for a further 3 hr. For qualitative experiments, the solution was partly neutralised with barium carbonate, centrifuged and deionised completely by passing the supernatant solution through Amberlite 1R-125(H) and Amberlite 1R-4B(OH) columns. For quantitative experiments the hydrolysate was neutralised with 2N-NaOH (phenolphthalein) and the solution made very slightly acid before estimation of glucose with Somogyi reagent.

In preliminary experiments, the formic acid was removed, after the first stage of the hydrolysis, by evaporation to dryness. However, the solid product remaining was insoluble in 2N-H_2SO_4 and the above procedure was therefore adopted.

Estimation of Formaldehyde.

The method was based on that of MacFadyen, Watkins and Anderson (1945) as modified by Parrish (1959). Samples (1 ml.) of the periodate oxidation mixture were withdrawn and 12·6% (w/v)

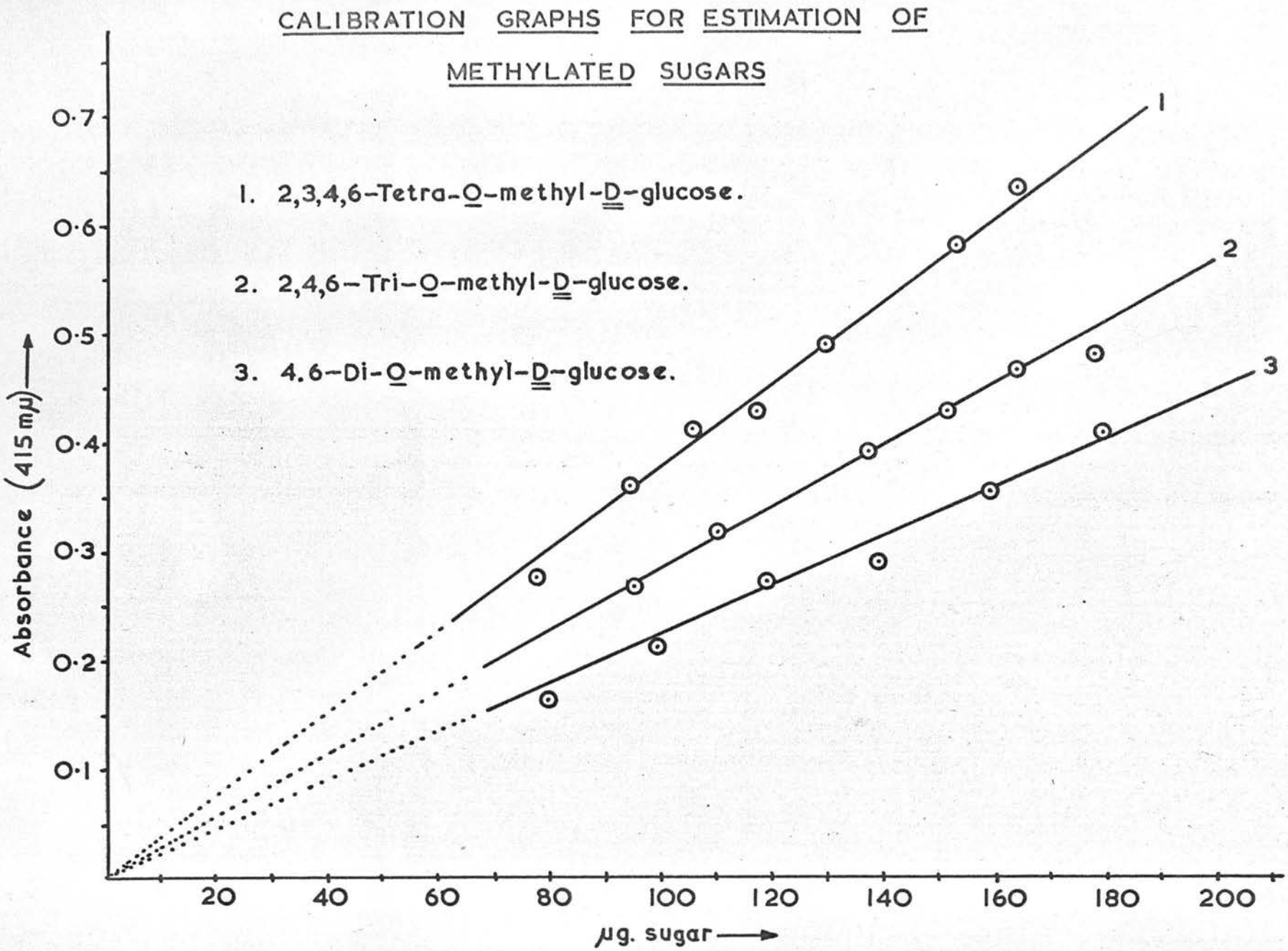

CALIBRATION GRAPHS FOR ESTIMATION OF METHYLATED SUGARS
1. 2,3,4,6-Tetra-O-methyl-D-glucose.
2. 2,4,6-Tri-O-methyl-D-glucose.
3. 4.6-Di-O-methyl-D-glucose.
Absorbance (415 mμ)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
20
40
60
80
100
120
140
160
180
200
μg. sugar
1
2
3

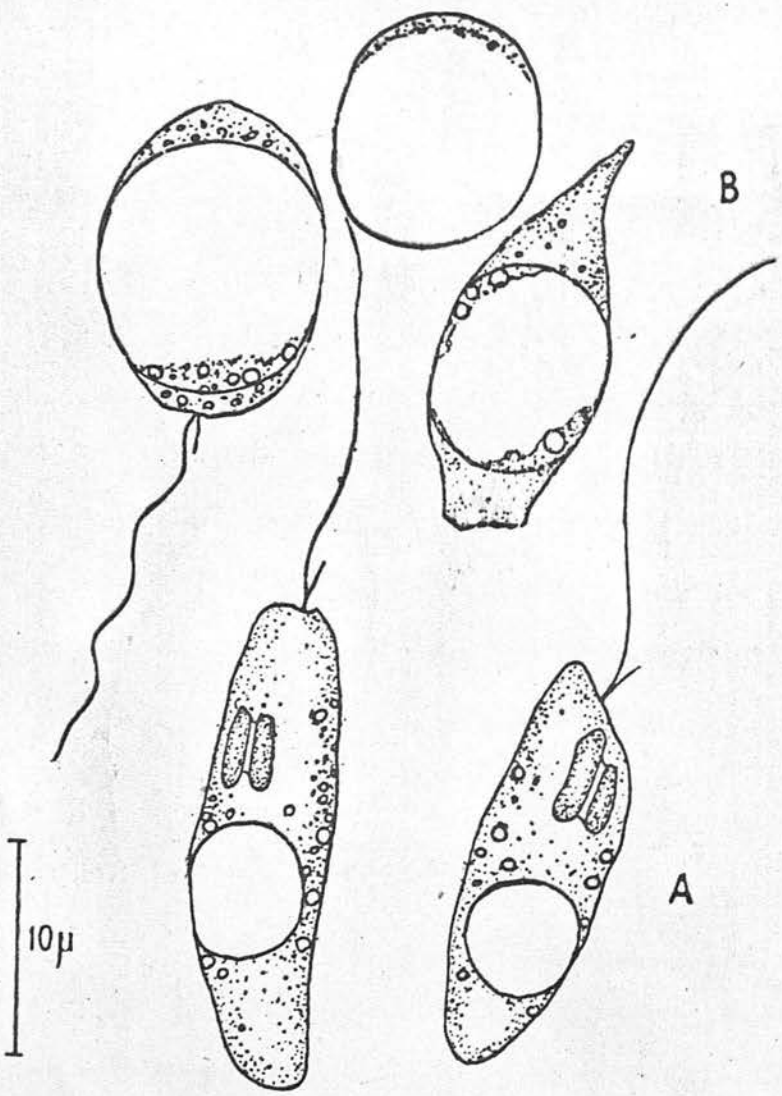
FIGURE 9

sodium sulphite (1 ml.) and ethanol (5 ml.) added. The mixture was left at 2° overnight (to ensure that all the polysaccharide material had precipitated) and then centrifuged. Samples (1 ml.) of the supernatant solution were heated with chromotropic acid reagent (9 ml. of a solution containing 0·5 g. of the sodium salt of chromotropic acid dissolved in 50 ml. of water and 200 ml. of 25N-H_2SO_4) at 100° for 30 min. The solution was cooled and 4·6% (w/v) thiourea (2 ml.) added. The absorbance of the solution was measured in 2 cm. cells at 570 mμ, using a Spectrophotometer SP600, against the corresponding blank, prepared by adding the sodium sulphite to the periodate before mixing with the polysaccharide solution. Calibrations were carried out, using standard formaldehyde solutions, under the same conditions as were used for the oxidation mixtures. Estimation of formaldehyde solutions for the calibration was carried out by the iodometric method described in "Practical Organic Chemistry" by Mann and Saunders, p.361. The ratio μg. formaldehyde : absorbance was generally in the region of 10·7:1.

Estimation of Methylated Sugars.

The method was exactly as described in chapter 1, p.12 . The calibration graphs for 2,3,4,6-tetra-O-methyl-D-glucose; 2,4,6-tri-O-methyl-D-glucose; and 4,6-di-O-methyl-D-glucose are shown in figure 9.

OCHROMONAS MALHAMENSIS



A, glucose 0.1% + Bacto tryptone 0.1% + Oxoid liver extract 0.1%;
B, glucose 2% + Bacto tryptone 0.2% + Oxoid liver extract 0.05%.
The quantity of leucosin in B is much larger than in A.

FIGURE 10

Section C

STUDIES ON THE RESERVE POLYSACCHARIDE FROM OCHROMONAS MALHAMENSIS

Introduction

Ochromonas malhamensis is a freshwater organism with two unequal flagellae, belonging to the order Chrysomonadina, a group whose members characteristically store oil and leucosin; the former occurs as droplets scattered throughout the cytoplasm, and leucosin accumulates in a posterior vacuole, which may be so large as to almost fill the cell. The organism, about 6μ x 10μ in size, is phagotrophic, i.e. can ingest particulate food, and, although it has a golden-brown chromatophore, it can only partially meet its needs by photosynthesis. In pure cultures, carbon requirements are satisfied by sugars, but not by acetate. The biology of Ochromonas malhamensis is reviewed by Pringsheim (1952). Figure 10 shows the main features of the organism.

The organisms were grown by Dr. J.F. Ryley and the polysaccharide material extracted and fractionated by Dr. A. R. Archibald. Part of the present investigation is concerned with the characterisation of the constituent monosaccharides of "fraction A," which were previously tentatively identified as glucose, galactose and mannose by chromatographic methods only. Fraction A was isolated (by Dr. Archibald) from the polysaccharide material obtained by addition of ethanol to the aqueous extracts of the cells. The material was

treated with Cetavlon and fractionated with acetone at concentrations of 0-55, 55-74, 74-80% acetone, these fractions being termed A, B and C respectively.

The major part of the present studies are concerned with "leucosin III." Previous work had shown that leucosin III consisted of glucose units linked by β-(1→3)-glucosidic linkages (Archibald, Manners and Ryley, 1958), but there was some doubt as to whether the molecules contained a low degree of branching or a few (1→6)-inter residue linkages. The present experiments, using the Smith degradation procedure (see figure 15) suggest that a low degree of branching is probably present. Further evidence for the presence of β-(1→3)-linkages in leucosin III is also presented.

Leucosin III was isolated (by Dr. A.R. Archibald) from the polysaccharide fraction B obtained above by addition of acetone to a cetavlon treated cell extract. Fraction B was refractionated into two portions with acetone 0-60 and 60-82% by vol. and the latter portion purified by dialysis, filtration and precipitation with ethanol to yield "leucosin III."

Experimental.

Examination of Fraction A. 1. Characterisation of the component monosaccharides.

Fraction A (0.7 g.) was heated at 100° for 2 hr. with 2N-H_2SO_4 (70 ml.). Neutralisation was carried out with barium carbonate and, after centrifuging down the barium sulphate, the solution was

evaporated to a volume of about 5 ml. Part of the hydrolysate (4 ml.) was applied to a column of Dowex 50W (x8; 200-400 mesh: Ba^{++} form) resin and the monosaccharides eluted with distilled water (Jones and Wall, 1960). The column was adjusted, by means of a movable syphon-head arrangement, to give one fraction (3-4 ml.) every 6-8 min. Fractions were analysed by paper chromatography, using 10:4:3 as developing solvent and aniline oxalate spray reagent. Fractions 30-34 contained glucose, 36-40 contained glucose and galactose, 42 contained these sugars and a trace of xylose, 44 also contained mannose, and 46-47 contained the three hexoses. Concentration of fractions 29-34 gave D-glucose, characterized as the β-penta-acetate (m.p. and mixed m.p. 130-131°); from fractions 39-41, D-galactose was obtained as the crystalline 1-methyl-1-phenylhydrazone (m.p. and mixed m.p. 180-183°). Fractions 44-50 were applied to Whatman 3MM paper, developed in 10:4:3 as solvent and the D-mannose section cut out and eluted with water. The D-mannose was characterised as the phenylhydrazone (m.p. and mixed m.p. 190-193°). Glucose penta-acetate was prepared by the method described in "Elementary Practical Organic Chemistry" by A. I. Vogel, Part 2, p.393, and the mannose and galactose derivatives by procedures described in Modern Methods of Plant Analysis, Vol.2, p.36.

2. Determination of the ratio of the component monosaccharides.

The molar proportion of the three hexoses was determined by the method of Wilson (1959). The total hydrolysate of Fraction A

(1 ml.) was diluted with water to a volume of 10 ml. and spots (5 µl. each) were applied to Whatman No.1 paper using a Shandon 2060 micropipette. The spots were dried and respotting carried out several times to give a gradation of quantities. In preliminary experiments, it was found that the quantity of mannose was so small that its estimation could not be carried out at the same time as the individual estimations of galactose and glucose. With 10:4:3 as solvent, if enough of the mixture was applied to the paper to give a measurable amount of mannose, the quantity of glucose present was such that it merged into the mannose spot. Using 9:1:1 as solvent the mannose was well separated from glucose and galactose even if the latter two hexose were present in very large amounts, but glucose and galactose were not separated from each other. The following procedure was therefore adopted.

Successive spotting to give 15-30 µls. of the diluted hydrolysate per spot was applied accurately to chromatography paper. Each paper was also spotted with known quantities of standard glucose and galactose solution. The chromatograms were developed in 10:4:3 and dried. The colour development of the spots and their subsequent estimation was carried out using the aniline hydrogen phthalate method described by Wilson (1959). The diluted hydrolysate of Fraction A contained glucose (4·77 µg./µl.) and galactose (0·734 µg./µl.), the amount of mannose being too small to be estimated with any accuracy.

In a second series of experiments 70-100 µl. of the diluted hydrolysate per spot were used and the chromatograms developed with

9:1:1 for 2·5 days. Standard solutions of glucose, galactose and mannose were spotted on the same paper as the unknown mixture. Since the weights of glucose and galactose per μl. were found from the first series of experiments, only the mannose was estimated. The ratio of glucose + galactose : mannose was 99·3:0·7, giving the following composition for the mixture:- glucose 86%, galactose 13%, mannose 1%.

Examination of Leucosin Preparation III

Polyglucose Content. Leucosin (14·0 mg.) was hydrolysed with sulphuric acid, neutralised and the solution diluted to 25 ml. Portions were taken for Somogyi estimation of glucose. The concentration of glucose was 0·567 mg./ml. corresponding to a polyglucose content of 91%. All subsequent weights of leucosin quoted are corrected using this value.

Qualitative Chromatographic Examination of Leucosin

A neutralised hydrolysate of leucosin was examined by paper chromatography using 10:4:3 and 9:1:1 as solvents. Chromatograms were run in each solvent in triplicate so that three spray reagents (silver nitrate, aniline oxalate and periodate-permanganate) could be applied. In the six chromatograms thus produced, only glucose was detectable in the leucosin hydrolysate. In a parallel experiment with a laminarin hydrolysate, mannitol (as well as glucose) was detectable using 9:1:1 as solvent and periodate-permanganate spray. Using various mixtures of glucose and mannose,

PERIODATE OXIDATION OF SOME β-(1→3)-GLUCANS

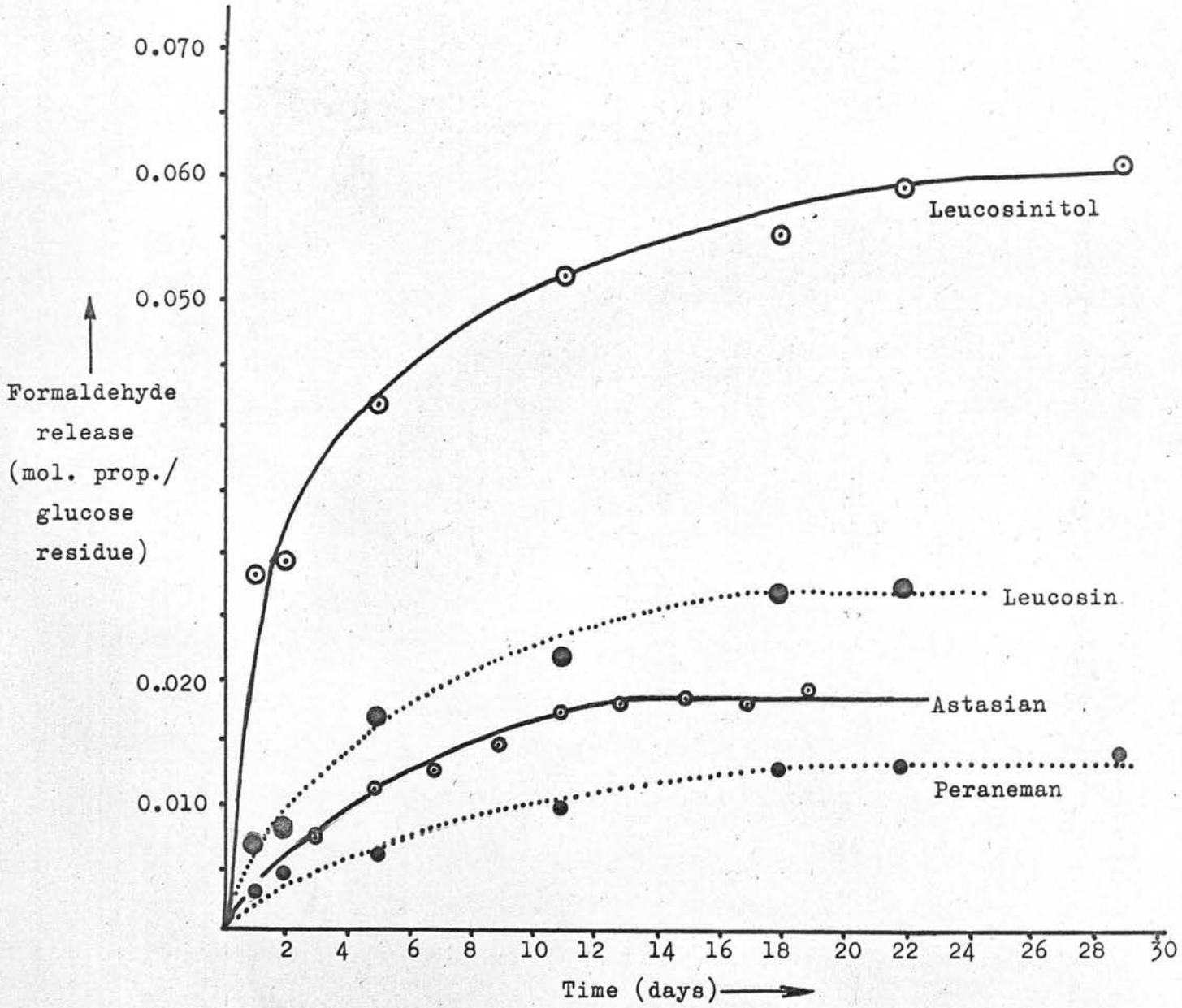


FIGURE 11

and 9:1:1 as solvent, mannose could be detected in concentrations of less than 1% (with respect to glucose) using aniline oxalate spray, but using silver nitrate spray even 2% mannose could not be detected.

Periodate Oxidation of Leucosin at Room Temperature (17°).

Leucosin (36·8 mg.) was oxidised with 0·3M-sodium meta-periodate (1 ml.) in a total volume of 10 ml. Samples (1 ml.) were withdrawn at intervals for estimation of formaldehyde as described in the experimental methods section. The apparent degree of polymerisation was calculated on the assumption that each reducing end-group yields one molecule of formaldehyde.

Time of oxidation (days)	Formaldehyde release*	Apparent degree of polymeri-sation	Time of oxidation (days)	Formaldehyde release*	Apparent degree of polymeri-sation
0·3	0·0057	176	11	0·0215	47
1	0·0069	145	18	0·0267	37
2	0·0080	126	22	0·0272	37
5	0·0169	59	29	0·0283	35

* The formaldehyde release is expressed in mol.prop./glucose residue.

These results are graphically represented in Figure 11.

Periodate Oxidation of Leucosin at 2°.

Leucosin (33·9 mg.) was oxidised as described above except that the solution was kept at 2°. After 22 and 29 days, small quantities of formaldehyde could be detected but at no time did the formaldehyde liberated exceed 0·005 mol.prop./glucose residue.

Preparation of Leucosinitol.

Leucosin (200 mg.) was dissolved in water (10 ml.) and potassium borohydride (100 mg.) added. The solution was agitated gently for 48 hr. and the excess potassium borohydride decomposed with acetic acid. The leucosinitol was precipitated with ethanol and washed thoroughly with 70% ethanol before drying in the usual manner.

Polyglucose Content of Leucosinitol.

Leucosinitol (8·9 mg.) was hydrolysed, neutralised and diluted to 25 ml. The concentration of glucose (estimated by Somogyi reagent) was 0·338 mg./ml. corresponding to a polyglucose content of 85·5%. Since all the reducing end-groups have been converted into sorbitol residues this value does not give the true purity of the sample. When allowance is made for the sorbitol residues, on the assumption that the degree of polymerization is 36, the purity of the sample is 88%. All subsequent weights of leucosinitol quoted are corrected using this value.

Periodate Oxidation of Leucosinitol.

Leucosinitol (26·6 mg. for the experiment at room temperature and 29·7 mg. for the experiment at 2°) was oxidised with 0·3M-sodium metaperiodate (1 ml.) in a total volume of 10 ml. Samples (1 ml.) were withdrawn at intervals for determination of formaldehyde. The apparent degree of polymerisation was calculated on the assumption that each sorbitol end-group yields 2

molecules of formaldehyde.

Time of oxidation (days)	17° Formaldehyde release*	17° Apparent degree of polymerisation	2° Formaldehyde release*	2° Apparent degree of polymerisation
0·3	0·0270	75	0·0191	105
1	0·0281	71	0·0236	85
2	0·0291	69	0·0255	78
5	0·0418	48	0·0280	71
11	0·0519	37	0·0393	51
18	0·0576	35	0·0484	42
22	0·0590	34	0·0520	39
29	0·0614	33	0·0540	38

* Formaldehyde release is expressed in mol.prop./glucose residue.

The results from the oxidation at 17° are indicated graphically in Figure 11 facing p.74.

Periodate Oxidation of Sorbitol at 2° and at room temperature.

The following experiments were carried out for comparative purposes. Sorbitol (2·16 mg.) was oxidised with 0·3M-sodium metaperiodate (1 ml.) in a total volume of 25 ml. Samples (1 ml.) were taken for estimation of formaldehyde. Experiments were carried out both at room temperature and at 2°.

Time of oxidation (days)	2° Formaldehyde release*	17° Formaldehyde release*
0·04	1·43	1·61
0·25	1·66	1·77
1	1·70	1·83
4·5	1·81	1·91
8	1·90	1·95
12	1·93	2·06
16	1·90	2·03

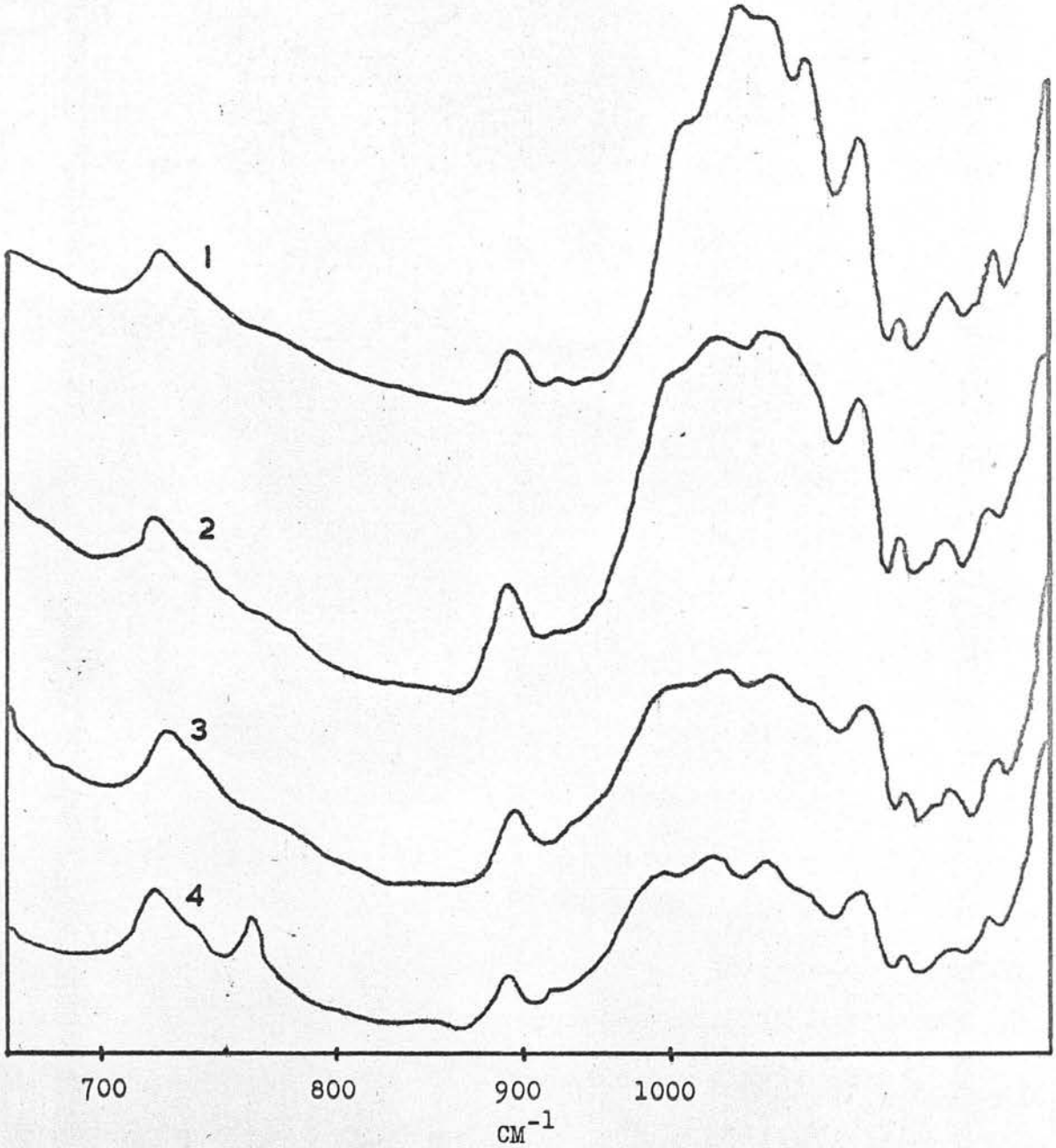
* Formaldehyde release is expressed in mol.prop./mol. sorbitol.

These results indicated that, although the release of formaldehyde from sorbitol at 2° is slow, the yield is in close agreement with the theoretical value of 2·00 mol.prop. if the oxidation is prolonged.

Overoxidation of Leucosin.

Leucosin (10·45 ml.) was dissolved in 0·1M-phosphate buffer of pH8 (12·5 ml.), 0·3M-sodium metaperiodate (2 ml.) added and the solution diluted to 25 ml. All volumes were measured at 37° and the oxidation mixture maintained at this temperature. Portions (1 ml.) were withdrawn at intervals for estimation of formaldehyde. A parallel experiment was carried out with laminarin (sample BB2). In each case the formaldehyde release was constant after about 30 hr. and amounted to 0·50 mol.prop./glucose residue from leucosin and 0·54 mol.prop./glucose residue from laminarin.

INFRARED SPECTRA OF SOME β-GLUCANS



1. Astasian
2. Laminarin
3. Leucosin
4. Chrysolaminarin

FIGURE 12

Infra-red Spectrum.

The infra-red absorption spectrum of leucosin was examined in Nujol with a Perkin-Elmer Infra cord spectrophotometer. The spectrum showed an adsorption band at 890 $cm.^{-1}$, and was almost identical with that of laminarin, and with that of a sample of chrysolaminarin provided by Dr. E. E. Percival (cf. Beattie, Hirst and Percival, 1961). The infra-red spectra of some polysaccharides are compared in Figure 12.

Action of Rhizopus arrhizus Enzyme Preparation on Leucosin.

The Rhizopus arrhizus preparation was the same as that used by Cunningham (1961), and had strong laminarinase activity. Leucosin (18·10 mg.) and enzyme preparation (4 mg.) were dissolved in 0·05M-citrate buffer of pH 4·8 (3 ml. and 2 ml. respectively) and the two solutions mixed. The digest was covered with a layer of toluene and incubated at 37° (all volumes being measured at this temperature). Samples (0·5 ml.) were removed at intervals for estimation of reducing power (as glucose) using the Somogyi reagent. An identical experiment was carried out using laminarin (sample BB2). Small samples were withdrawn for chromatographic examination using 6:4:3 as solvent and silver nitrate spray reagent. After 3 hr., glucose (+++), laminaribiose (+) (R_G-value 0·85) and another reducing product of R_G 0·53 (±) were detected in both the leucosin and in the laminarin digests. With subsequent samples it became increasingly difficult to detect the laminaribiose.

Results

Time of incubation (hr.)	Laminarin: Apparent conversion into glucose(%)	Leucosin: Apparent conversion into glucose(%)
1	45·0	-
4	61·7	52·0
20	70·5	66·0
45	71·2	72·2
72	70·5	73·5
94	-	74·8

Smith Degradation of Leucosin.

Leucosin (99 mg.) was oxidised with 0·3M-sodium metaperiodate solution (3 ml.) in a total volume of 25 ml. for 48 hr. at 18°. Excess of periodate was destroyed by addition of 12·6% (w/v) sodium sulphite solution (12·5 ml.), and, after 30 min., potassium borohydride (100 mg.) was added. After 40 hr. at 18°, the excess of borohydride was decomposed by careful addition of dilute acetic acid to give pH 6-7. 2N-H_2SO_4 (5 ml.) was added, the solution diluted to 100 ml. with distilled water (making the solution 0·1N with respect to H_2SO_4) and the mixture left at 18° for 24 hr. to effect cleavage of any acetal linkages, which may have been formed. (The general conditions are similar to those employed in parallel studies on laminarin by Smith and Unrau, 1959). The solution was neutralised with sodium hydroxide to pH 7·0 and dialysed for 48 hr. against five successive changes of distilled water (3 l. each).

The non-diffusible material was recovered by freeze-drying.

The material (yield 114 mg.) had a polyglucose content of 79%, equivalent to a 91% recovery of polysaccharide. The impurity was inorganic material, but iodate ions were absent (these would have interfered with Somogyi estimation).

Undiffusible polysaccharide (36·3 mg., based on polyglucose content) was oxidised with 2mM-sodium metaperiodate (5 ml.) at 2°. Samples (1 ml.) were removed at intervals, and treated with 12·6% (w/v) sodium sulphite solution (0·5 ml.) and ethanol (6 ml.) to remove respectively excess of periodate and polysaccharide, and the formaldehyde contents of the supernatant solutions were measured by the chromotropic acid procedure. The production of formaldehyde after 2, 5·5 and 24 hr. amounted to 0·0062, 0·0059 and 0·0058 mol.prop./glucose residue respectively. A molecule containing only one internal (1→6)-glucosidic linkage would yield about 0·03 mol.prop. of formaldehyde. Using the same periodate oxidation conditions, glucosylmannitol from a partial acid hydrolysate of laminarin (obtained from Dr. D. H. Hutson) yielded 0·96 mol. prop. of formaldehyde (theory, 1·00 mol. prop.; for details see chapter 3, p.161).

Discussion.

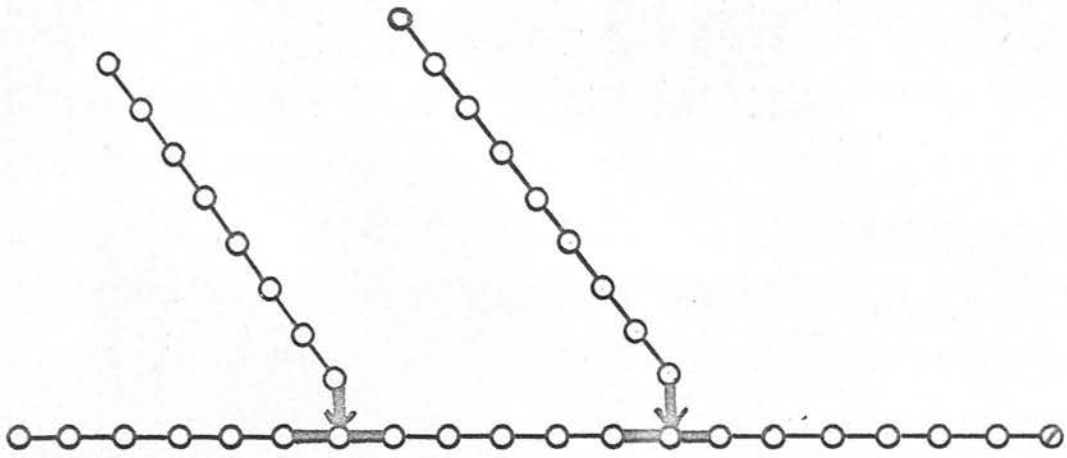
In the present investigation the constituent monosaccharides of Fraction A (the first fraction obtained by addition of acetone to the cetavlon purified extract) were separated and characterised as D-glucose, D-galactose and D-mannose by means of crystalline

derivatives. The presence of a trace quantity of D-xylose was also indicated. This would suggest that a highly complex system of carbohydrate synthesising enzymes is present in Ochromonas malhamensis. This is in contrast to Euglena gracilis (Clarke and Stone, 1960), Peramena trichorphum (see section D) and Astasia ocellata (see section E) which possess D-glucose as the sole monosaccharide constituent of their respective polysaccharides. The relative amounts of the three hexoses present in a hydrolysate of fraction A, as estimated by chromatographic techniques, were glucose 86%, galactose 13% and mannose 1%. To ascertain whether fraction A is a single polysaccharide or a mixture of several polysaccharides would require a linkage analysis by partial acid hydrolysis; unfortunately this would require about 10 g. of material.

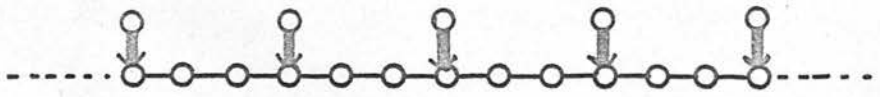
Leucosin III which had been purified by cetavlon and acetone precipitations, possessed D-glucose as the only monomer unit. Extensive chromatographic examination of a hydrolysate of this fraction was performed using several solvents and spray reagents; mannitol and mannose were absent.

Dr. A. R. Archibald showed that leucosin has a low specific rotation, suggesting the presence of β-glucosidic linkages. In the present studies the infra-red spectrum of leucosin, showing a characteristic peak at 890 $cm.^{-1}$ and the enzymic degradation of leucosin to laminaribiose and glucose by an exo-β-glucosidic preparation from Rhizopus arrhizus confirmed the presence of β-glucosidic linkages. The action of the enzyme is random and its specificity may be defined by

STRUCTURES OF SOME β-GLUCANS

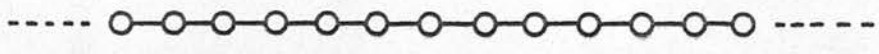


(a) A possible structure for leucosin
(from Ochromonas malhamensis.)



(b) Structure of a glucan from Fungi imperfecti

Smith
degradation



(c)

Key:- O—O ,glucose residues linked by a β-(1→3) linkage.

O ,glucose residues linked by a β-(1→6) linkage.

All thick lines represent linkages which may be resistant to attack by Rhizopus arrhizus enzyme preparation.

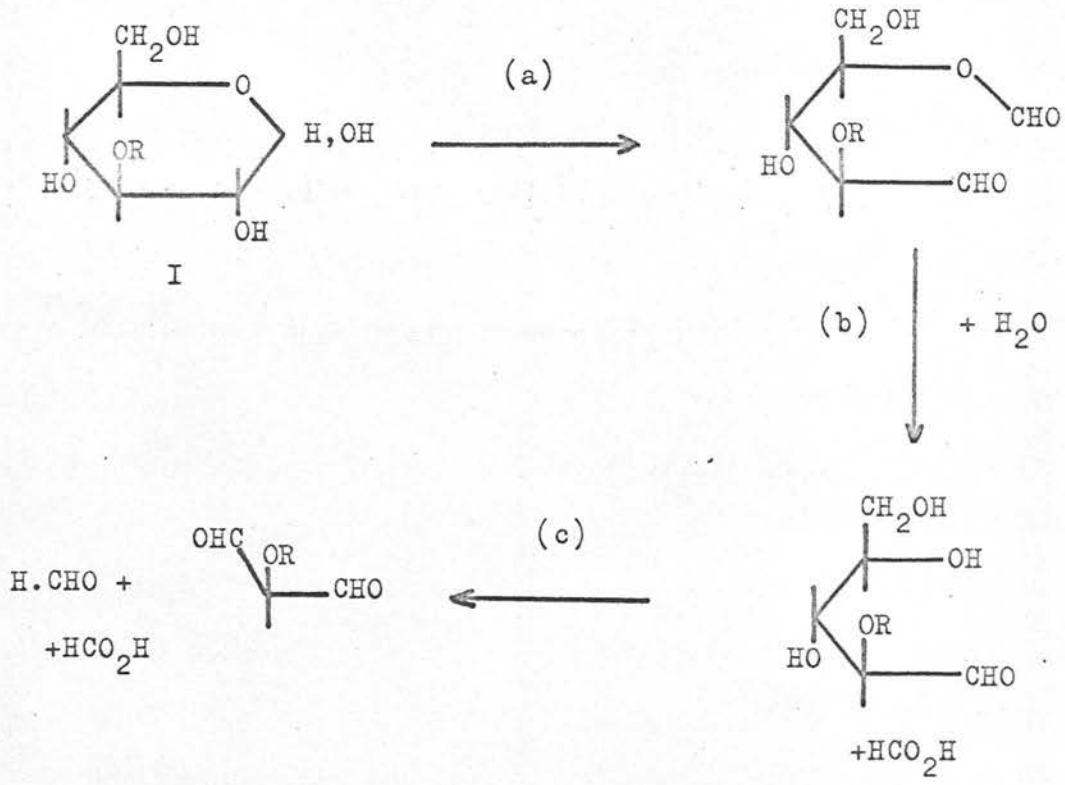
FIGURE 13

$$-\text{G}\overset{(a)}{-}\text{G}\overset{(b)}{-}\text{G}-$$

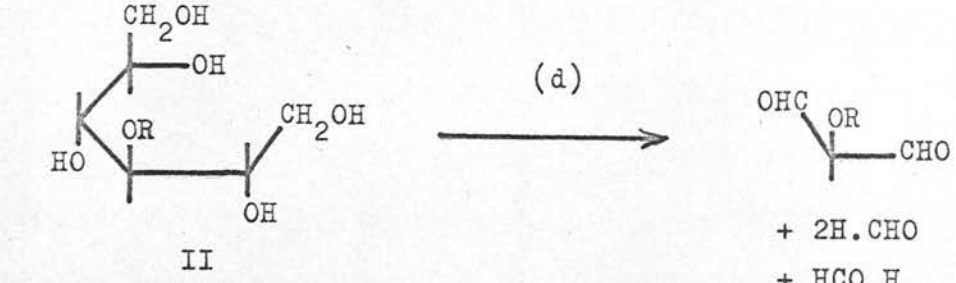
where G represents a β-D-glucopyranose residue, (a) a (1→3)-linkage, and (b) the linkage hydrolysed, which may be a (1→3) or a (1→4)-linkage, Cunningham and Manners (1961). Johnson et al. (1963) in their studies on the glucan from Fungi imperfecti, claim that the preparation will not hydrolyse a (1→6) branch point, but that it can hydrolyse the adjacent (1→3) linkages. The "branch" in the glucan was, however, only one glucose unit long (see figure 13). The extent to which the enzyme can hydrolyse linkages adjacent to a branch point, where the branch contains several glucose units is not known. Dr. A. R. Archibald obtained an average chain length of 13 glucose residues by periodate oxidation (for details see later). Since the degree of polymerisation is 36 glucose residues, the molecules, on the average, contain two branch points per molecule. The extent of hydrolysis by Rhizopus arrhizus preparation can be explained by assuming that the (1→3)-linkages on either side of the glucose unit, which forms the branch point, cannot be hydrolysed. If the enzyme action is limited in this manner, the extent of hydrolysis would be about 80% (cf. experimental value of 75% after 94 hr.). This is depicted diagrammatically in figure 13.

In experiments carried out by Dr. A. R. Archibald, the formic acid produced on periodate oxidation of leucosin was 1 molecule per 12·8 glucose residues, and the reduction of periodate was 0·15 mol.prop. after 24 hr., confirming the presence of a large number of (1→3) linkages, the presence of which was suggested by partial

REACTION OF PERIODATE ON VARIOUS REDUCING-END GROUPS



I



II

(e)

$$RO.CH_2\left[CH(OH)\right]_4.CH_2.OH \longrightarrow RO.CH_2CHO + 3H.CO_2H + H.CHO$$

III

FIGURE 14

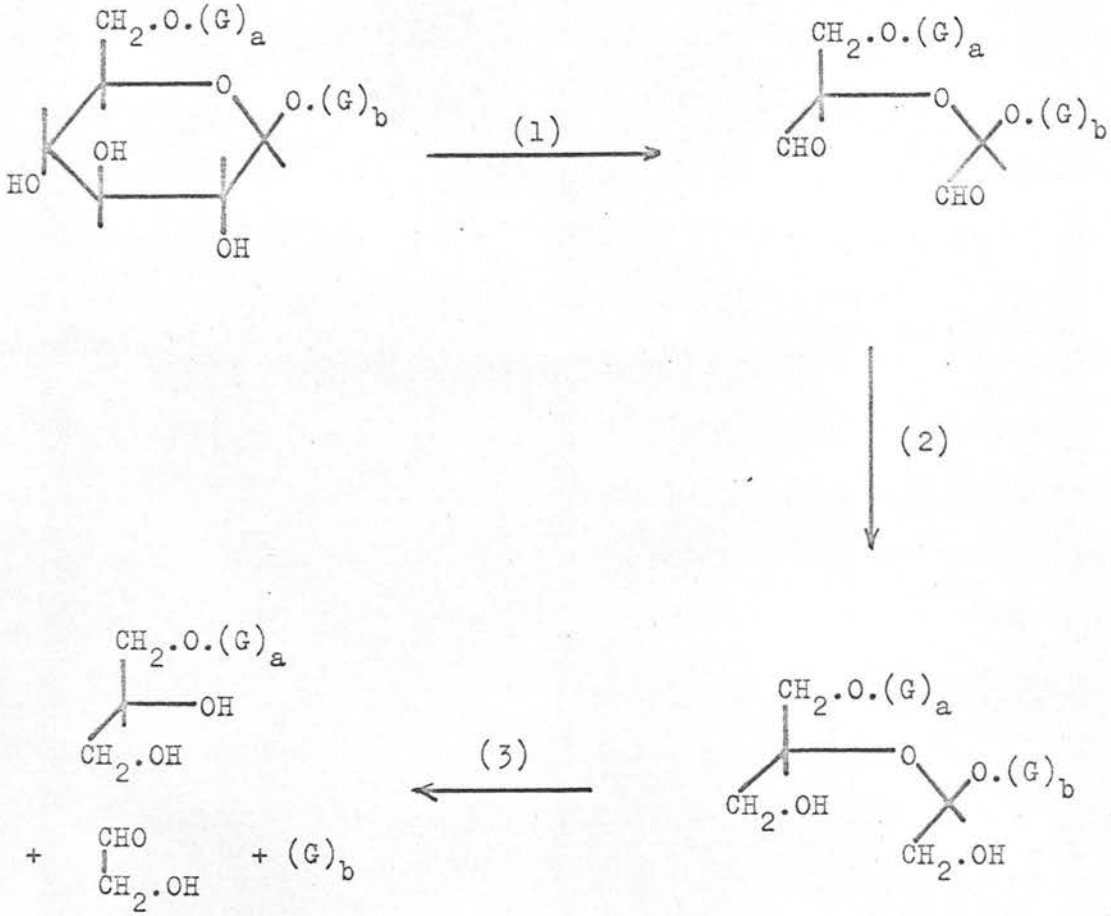
acid hydrolysis. In the present investigation, the final production of formaldehyde on periodate oxidation at room temperature was 0·028 mol.prop./glucose residue from leucosin and 0·061 mol.prop./glucose residue from leucosinitol. On periodate oxidation at 2° the release of formaldehyde was negligible and from leucosinitol amounted to 0·054 mol.prop./glucose residue. Figure 14 illustrates the action of periodate at the reducing end of some glucans, where R represents a chain of glucose units linked by β-(1→3)-linkages. Reactions (a), (d) and (e) can take place at 2°, but for reaction (b) to proceed, room temperature is required. Hence at 2° a terminal glucose residue will yield no formaldehyde. Leucosin itself is therefore devoid of any sugar alcohol groups in the reducing position; this is in agreement with the examination of the total acid hydrolysate of leucosin, which contained only glucose. In contrast to this, about half the molecules in laminarin are terminated by a mannitol end-group as indicated in structure III (Anderson, Hirst, Manners and Ross, 1958). At room temperature reactions (a), (b) and (c) take place and one molecule of formaldehyde is liberated per molecule. On this basis the average degree of polymerisation of leucosin is about 34-36 glucose residues. Reduction of leucosin will produce a structure of type II (i.e. the reducing glucose end group is converted into a sorbitol group), which, on periodate oxidation at 2° would be expected to yield 2 molecules of formaldehyde per polysaccharide molecule. The formaldehyde liberated from leucosinitol at room temperature corresponded to a degree of

polymerisation of about 33-35 glucose residues. However, at 2° the liberation of the second molecule of formaldehyde from the sorbitol group is very slow (cf. sorbitol itself at 2° gives 1·70 and 1·90 mol.prop. after 1 day and 16 days respectively) and the reaction did not go to completion.

Periodate oxidation of a linear glucan, containing either (1→3) or (1→4)-glucosidic linkages or a combination of both linkages, at pH8 and 35° will yield one mol.prop. of formaldehyde/glucose residue (Hough and Perry, 1956). Experiments with lichenin and linear amylose gave values of 0·98 and 0·92 and prop. formaldehyde/glucose residue respectively. The yield from leucosin was 0·50 mol.prop., suggesting that the molecule possesses a small proportion of linkages other than (1→3). The average chain length of 12·8 glucose residues and the average degree of polymerisation of 33-36 confirm the presence of other linkages and indicate the presence of three "triol" groups per molecule, which may be present as (a) non-reducing end groups, implying the presence of two branch points/molecule, (b) two internal (1→6)-glucosidic linkages/molecule or (c) one branch point and one internal (1→6)-glucosidic linkage/molecule. Structures of the (b) and (c) type exist in yeast glucan (Peat, Whelan and Edwards, 1958) and chrysolaminarin (Beattie, Hirst and Percival, 1961).

It is unfortunate that the solution of this problem by methylation or partial acid hydrolysis requires larger quantities of leucosin than were available. Nevertheless, additional periodate-oxidation experiments indicate that inter-chain linkages

DEGRADATION OF AN INTERNAL (1→6)-LINKED GLUCOSE RESIDUE



Degradation of an internal (1→6)-linked D-glucose residue by (1) periodate oxidation followed by (2) borohydride reduction and (3) partial hydrolysis.

$(G)_a$ or $(G)_b$, chain of (1→3)-linked glucose residues.

FIGURE 15

rather than (1→6)-glucosidic linkages are most probably present (figure 15).

Leucosin was subjected to the Smith degradation procedure (see Smith and Montgomery, 1959) involving periodate oxidation, borohydride reduction and partial acid hydrolysis. The residual polysaccharide was non-diffusible and, on further oxidation with a dilute solution of sodium metaperiodate, the yield of formaldehyde was only one-fifth of that expected from structure (c), and one tenth of that from structure (b). If leucosin contained internal (1→6)-glucosidic linkages, then the degradation procedure would yield linear chains of degree of polymerisation 12-17. Since laminarin preparations with a degree of polymerisation 20-25 are diffusible (W. D. Annan and D. J. Manners, unpublished work), any linear chains derived from leucosin should also be diffusible.

Moreover, mild hydrolysis of the leucosin polyalcohol would yield a 1-O-substituted glycerol (figure 15) which on controlled periodate oxidation would yield 1 mole of formaldehyde/molecule. The observed yield was only 0·204 mole/molecule, whereas in a control experiment with 1-O-β-glucosylmannitol 0·96 mole of formaldehyde/mole was liberated. The small yield of formaldehyde could arise from a limited reaction at the residue of the original reducing group. Furthermore, since the "Smith" degraded polysaccharide is soluble in cold water, it is thought that leucosin contains true "branches" and not single glucose residue "stubs," such as are present in the glucan from Fungi imperfecti (see figure 13).

The above evidence that leucosin is a β-(1→3)-glucan with, on the average, two branch points per molecule, is in agreement with its physical properties. Leucosin dissolves readily in water and will remain in solution indefinitely. The "insoluble" form of laminarin, although it has a degree of polymerisation of only 24 glucose units (Anderson et al., 1958) precipitates spontaneously from solution. Recent studies have shown that the "soluble" form of laminarin has a higher degree of branching than the "insoluble" form (M. Fleming and D. J. Manners, unpublished work).

Section D

STUDIES ON THE RESERVE POLYSACCHARIDE FROM PERANEMA TRICHOPHORUM

Introduction

Peranema trichophorum is a colourless member of the order Euglenoidina. It lives in ponds rich in organic matter, and feeds phagotrophically on a variety of plant and animal food, e.g. other protozoa such as Ochromonas. It is an elongate organism, approximately 55μ x 22μ in size in culture, that can move either by swimming or gliding; it has a stout flagellum about as long as the body projecting anteriorally, and another more delicate flagellum extending spirally backwards adhering to the pellicle. Paramylon is stored in the form of flat granules about 3·4μ diam. x 2·3μ thick. The biology of this organism is described in detail by Chen (1950).

The cultures of Peranema trichophorum were grown by Dr. J.F. Ryley and the extraction of the paramylon granules and preliminary investigations on their structure carried out by Dr. W.L.Cunningham (Cunningham, Manners and Ryley, 1961). In the present investigation, studies on the paramylon granules have been continued using a second batch of material isolated by Dr. Cunningham.

To avoid confusion between the paramylon from P.trichophorum and from Astasia ocellata (see section E) the former will be named

"peraneman" and the latter "astasian" and the corresponding derived alcohols "peranemitol" and "astasitol."

Experimental.

Polyglucose Content

Peraneman (18·4 mg.) was hydrolysed, as described in the experimental methods section, and the neutralised hydrolysate diluted to 50 ml. Glucose (0·397 mg./ml.) was produced, corresponding to a polyglucose content of 97·2%.

Periodate Oxidation of Peraneman at 17°.

Peraneman (36·9 mg.) was oxidised with 0·3M-sodium metaperiodate (1 ml.) in a total volume of 10 ml. at room temperature and the mixture shaken vigorously. Samples (1 ml.) were taken at intervals for estimation of formaldehyde. The apparent degree of polymerisation was calculated on the assumption that each reducing end-group yields one molecule of formaldehyde.

Time of oxidation (days)	Formaldehyde Release (mol.prop./glucose residue)	Apparent degree of Polymerization (glucose residues)
1	0·0031	320
5	0·0062	161
11	0·0097	103
18	0·0128	78
22	0·0130	77
29	0·0142	71

Periodate Oxidation of Peraneman at 2°.

The conditions of the oxidation were the same as described in the previous experiment except that the mixture was kept at 2°. A very small amount of formaldehyde was detectable in samples taken between 18 and 35 days but at no time did the amount liberated exceed 0·004 mol.prop./glucose residue.

Preparation of Peranemitol.

Peraneman (200 mg.) was suspended in water (10 ml.) and potassium borohydride (100 mg.) added. The mixture was shaken for 48 hr. and the excess borohydride decomposed with acetic acid. Ethanol (4 vol.) was added and the polysaccharide material centrifuged down, washed and dried.

Polyglucose Content of Peranemitol. Peranemitol (24·9 mg.) was hydrolysed, neutralised and diluted to exactly 50 ml. Estimations using Somogyi reagent indicated glucose (0·524 mg./ml.) corresponding to a polyglucose content of 94·6%.

Periodate Oxidation of Peranemitol at 17°.

The oxidation of peranemitol (25·2 mg.) was carried out as described for peraneman. The apparent degree of polymerisation was calculated on the assumption that each sorbitol end-group yields 2 molecules of formaldehyde.

Time of oxidation (days)	Formaldehyde release mol.prop./glucose residue	Apparent degree of polymerisation (glucose residues)
1	0·0057	350
5	0·0096	208
11	0·0167	121
18	0·0189	106
22	0·0213	94
29	0·0225	89
35	0·0228	88

Overoxidation of Peraneman.

To peraneman (9·45 mg.) was added 0·1M-phosphate buffer of pH8·0 (12·5 ml.), 0·3M-sodium metaperiodate (2 ml.) and water (10·5 ml.). The mixture was vigorously shaken at 37° and samples (1 ml.) taken at intervals for estimation of formaldehyde.

Time of oxidation (days)	Formaldehyde release mol.prop./glucose residue
5	0·223
19	0·314
22	0·314
25	0·309

The yield of formaldehyde in an identical experiment with pachyman was 0·365 mol.prop.

Action of Rhizopus arrhizus Enzyme Preparation on Peraneman.

Peraneman (ca. 20 mg.) was treated with 2N-NaOH; after five

hours most of the polysaccharide had dissolved. When the solution was neutralised it formed a viscous mass to which was added 0·05M-citrate buffer of pH 4·8 (1 ml.) and enzyme powder (4 mg.). The enzyme preparation was the same as that used by Cunningham (1961). The mixture was incubated at 37° and samples taken for direct spotting onpaper chromatograms. After 1 and 2 days, spots corresponding to glucose and laminaribiose were readily detected, using 6:4:3 as solvent and silver nitrate spray reagent. In subsequent samples only glucose was detected. A sample examined, before the addition of the enzyme preparation, contained no reducing sugars. Since the mixture was heterogeneous it was not possible to follow the reaction quantitatively.

Discussion.

The results of Cunningham, Manners and Ryley (1961) indicated that the reserve polysaccharide from Peranema trichophorum is a β-(1→3)-glucan. In the present investigation, the release of formaldehyde from the polysaccharide and from the corresponding alcohol indicate that the degree of polymerization is about 80 glucose residues. Since there was no significant release of formaldehyde from peraneman at 2°, the molecules must be devoid of mannitol-terminated reducing end groups, which are to be found in laminarin (see, Anderson, Hirst, Manners and Ross, 1958). This is in agreement with the fact that only glucose is detected on total acid hydrolysis of peraneman. The insolubility of

peraneman prevented a complete study of the extent of hydrolysis by Rhizopus enzyme preparation, but the clear indication that laminaribiose was present, in the enzyme digest, is further evidence for the presence of β-(1→3)-glucosidic linkages. It is thought that the insolubility of peraneman has prevented its complete overoxidation and the yield of 0·3 mol.prop. of formaldehyde shows only the existence of adjacent (1→3)-linked glucose residues near to the reducing end of the polysaccharide chain. Pachyman has been shown to be a linear polymer of β-(1→3) linked glucose residues (Warsi and Whelan, 1957) but the yield of formaldehyde from it was only 0·37 mol.prop./glucose residue.

The reserve polysaccharide from Peranema trichophorum resembles that of Euglena gracilis both in its solubility properties and in its molecular structure. The pattern of polysaccharide synthesis in Peranema is much simpler than in Ochromonas since the former contains only glucose as polysaccharide constituent. The principal properties of peraneman are compared with those of other, similar polysaccharides in Table 3 p.119.

Section E

STUDIES ON THE RESERVE POLYSACCHARIDE FROM ASTASIA OCELLATA

Introduction

Astasia ocellata is a colourless euglenoid flagellate. The euglenoid flagellates are unique, in that they are the only protozoa which synthesise paramylon (paramylum) granules as their reserve polysaccharide. The order Euglenida is separated into three families on the basis of the presence or absence of chlorophyll and stigma, and on the number of flagella. (A stigma is a red or orange structure often made up of small granules and it is part of the mechanism that causes certain flagellates to migrate towards light). The family Euglenidae includes those organisms which contain chlorophyll and a stigma; the families Astasiidae and Peranemidae contain those without chlorophyll or stigma, the former containing those with one flagellum and the latter those with two.

The species Astasia ocellata was first described by Khawkine (1886) as a free-living, permanently colourless euglenoid flagellate, with one flagellum. Khawkine also observed that Astasia ocellata possessed a stigma, thus making the organism an exception to the rule that members of the genus Astasia do not contain a stigma. Jahn and McKibben (1937) suggested that a

ASTASIA

(KHAWKINEA)

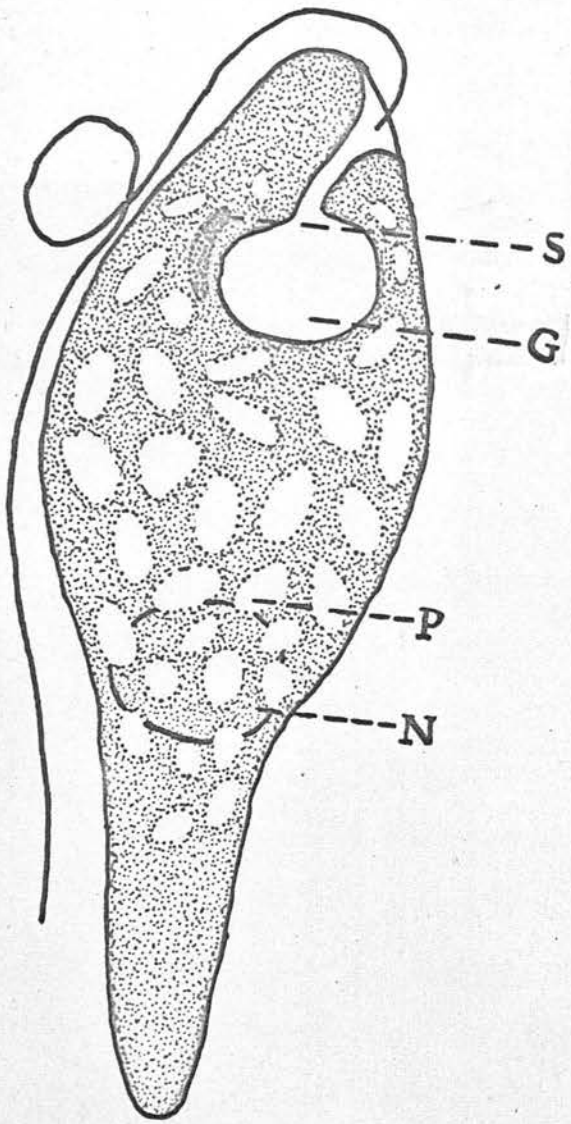


FIGURE 16

separate genus (Khawkinae) should be established within the family Euglenidae to include all stigma-bearing Astasia. Although the establishment of the new genus has been criticised by Pringsheim and Hovasse (1950), Astasia ocellata is sometimes referred to as Khawkine ocellata.

Jahn and McKibben (1937) described an organism which they called Khawkine halli (shown in figure 16). This organism is identical to Astasia ocellata except that the flagellum is only 1¼ times the body length compared with 1½ to 2 times the body length in the case of the Astasia ocellata described by Khawkine (1886). Both these organisms are about 30 to 60 μ in length by about 13-18 μ wide, generally bluntly rounded anteriorly and prolonged and sharply rounded posteriorly, although they may contract to a spherical or elongate and cylindrical form. The essential features are shown in figure 16. The stigma (S) is at the anterior end of the cell, close to the gullet (G). The nucleus (N) tends to be centrally located but may be freely carried to all parts of the cell during contraction. The paramylon bodies (P), which may vary in number from 25 to 100 in each organism, are generally elliptical or polyhedral (1 to 3 μ in size), although the smaller ones (0·5 μ) may be spherical. Astasia ocellata is a non-photosynthetic colourless counterpart of Euglena quartana.

The object of the present experiments was to isolate the paramylon granules from Astasia ocellata and to examine their chemical structure.

Experimental

Cultures of Astasia ocellata were grown by Dr. J.F. Ryley in a medium consisting of sodium acetate 0·1% (w/v), Oxoid lab lemco 0·1%; Difco yeast extract 0·2%, Difco Bacto tryptone 0·2% and calcium chloride 0·001%; as growth and acetate utilization resulted in a gradual increase in pH to a maximum around pH8, the initial pH of the medium was reduced to 5·5 to allow maximal growth over the tolerated pH range. Extremely poor growth was obtained when Oxoid yeast extract and tryptone were substituted for the Difco products. For bulk growth, batches of 25 2 l. conical flasks were used, each containing 500 ml. medium; incubation was at 24° in the dark for 6 or 7 days. Cells were harvested by centrifuging and washed and stored in methanol.

Isolation of the paramylon granules (astasian).

The method of extraction was essentially that of Clarke and Stone (1960). The cells were washed with ethanol, suspended in water and disrupted by ultrasonic vibrations. The cell debris, after washing with ethanol and ether and drying, weighed 16 g. This material was incubated with trypsin (0·4 g. of a Nutritional Biochemicals Corp. preparation in 400 ml. of 0·1M-sodium phosphate buffer, pH 7·6) at 37° for 40 hr., the mixture centrifuged, and the residue extracted three times with saturated urea solution and washed thoroughly with water. The off-white material was stirred in 0·1M-sodium chloride solution and centrifuged at 1,500 r.p.m. for 5 min. followed by 5 min. at 4,000 r.p.m. Two layers were formed; a highly compact, pure white bottom layer and a thin, loosely packed brown top layer of denatured protein, which was carefully scraped off with a spatula. This process was repeated. The white material was suspended in water (1,500 ml.) and shaken vigorously with toluene (150 ml.) for 24 hr. The mixture was centrifuged gently (1,000 r.p.m.) and the toluene, toluene-protein gel and the water removed. This process was repeated a further five times to yield a pure white solid, which weighed 8·8 g. on drying, corresponding to 55% of the dried cell material.

Preliminary Observations.

The product had the following properties:-

(a) resembled granular starch in its general physical appearance;

(b) did not stain with iodine;

(c) was insoluble in boiling water and in 2N-H_2SO_4 at 100°;

(d) dissolved after gentle agitation for 2-3 min. in N-NaOH;

(e) neutralisation of the alkaline solution of the material gave a gelatinous precipitate, which was insoluble in boiling water and in 2N-H_2SO_4 at 100°.

Total Acid Hydrolysis

The polysaccharide material (7 mg.) was hydrolysed successively with formic acid and sulphuric acid as described in the experimental methods section. The neutralised hydrolysate was examined by paper chromatography using 10:4:3, 4:1:5 and 6:4:3 as solvents and the aniline oxalate followed by silver nitrate spraying procedure (see p. 12). Glucose was the only sugar detected. The absence of mannitol and other sugar alcohols was indicated by running a chromatogram in 9:1:1 as solvent and periodate-permanganate spray; only glucose was detected. Verification that glucose was the only monomer unit present was obtained by incubating the hydrolysate with a Takamine DeO glucose oxidase preparation. A single spot corresponding to gluconic acid was produced using 10:4:3 and silver nitrate spray.

Partial Acid Hydrolysis

Astasian (10 mg.) was heated with 90% formic acid (1 ml.) at 100° for 1 hr. While the solution was still hot, 0·33N-H_2SO_4 (2 ml.) was added and heating at 100° continued for a further 0·5 hr. The hydrolysate was deionized by passing it slowly

through a Duolite A-4 anion resin column (25 cm. x 2.5 cm.). The product was compared chromatographically with a partial acid hydrolysate of laminarin (sample BB2) prepared by heating the polysaccharide (10 mg.) with 0·33N-H_2SO_4 (1 ml.) at 100° for 1 hr. and deionizing with Duolite A-4 resin.

Results

Solvent:

ethyl acetate/pyridine/water 10 : 4 : 3		n-butanol/ethanol/water 40 : 11 : 19	
R_G-values		R_G-values	
Astasian Hydrolysate	Laminarin Hydrolysate	Astasian Hydrolysate	Laminarin Hydrolysate
1.00	1.00	1.00	1.00
0.76	0.76	0.74	0.74
0.49	0.49	0.52	0.50
		0.37	0.36

Polyglucose Content

The dried polysaccharide (11·8 mg.) was weighed out accurately into a small tube and hydrolysed successively with formic acid and sulphuric acid as described in the experimental methods section. The neutralised hydrolysate was diluted to exactly 25 ml. and portions (3 ml.) taken for Somogyi estimation as glucose. Glucose (1·51 mg.) was detected, corresponding to a polyglucose content of 95·7%. All weights of astasian subsequently quoted are corrected using this value.

Infrared Spectrum.

The infrared-absorption spectrum of astasian was examined in Nujol with a Perkin-Elmer Infracord spectrophotometer. The spectrum which showed an absorption band at 890 $cm.^{-1}$, was identical to that of peraneman (see W.L. Cunningham, Ph.D. Thesis) and differed only very slightly from that of laminarin. The infrared spectra of various glucans are shown in figure 12 facing p.78.

Specific Rotation.

Astasian (101·5 mg.) was dissolved by 3 min. gentle agitation in 1N-NaOH and the rotation measured in a 1 dm. tube.
$[\alpha]_D^{20} = +17^\circ$.

Action of Rhizopus arrhizus Enzyme Preparation.

The solution used for determination of specific rotation was dialysed to remove sodium hydroxide. It was, however, found that a gelatinous precipitate started to form, before all the alkali had been removed, thus preventing a quantitative study of the enzyme action. After dialysing for 2·5 days, the gel-like suspension was shaken and a volume (3 ml.) containing 6-10 mg. of polysaccharide added to a solution containing Rhizopus arrhizus enzyme preparation (10 mg.) in 0·1M-citrate buffer, pH 4·8 (2 ml.). The mixture was covered with a layer of toluene and incubated at 37°. Paper chromatograms were spotted directly and a laminarin partial acid hydrolysate run as standard. On chromatographic examination of a 0·5 hr. sample spots corresponding to glucose (+++),

laminaribiose (++) and laminaritriose (+) were detected. An analogous experiment was carried out using paramylon from Euglena gracilis and similar results were obtained. With chrysolaminarin (a sample provided by Dr. E. E. Percival), which did not require alkaline pretreatment, as it was water-soluble, the same three spots were readily detectable. In all cases, on prolonged incubation the laminarisaccharides were destroyed and only glucose was detected. The solutions, prior to addition of enzyme, did not contain reducing sugars.

Periodate Oxidation of Astasian at 20°.

Astasian (104·1 mg.) was oxidised with 0·3M-sodium metaperiodate (2·5 ml.) in a total volume of 25 ml. at 20° and the mixture shaken vigorously. Samples (1 ml.) were taken at intervals for estimation of formaldehyde. The apparent degree of polymerisation was calculated on the assumption that each reducing end-group yields one molecule of formaldehyde.

Time of oxidation (days)	Formaldehyde release*	Apparent degree of polymerisation	Time of oxidation (days)	Formaldehyde release*	Apparent degree of polymerisation
1	0·00654	153	11	0·01722	58
3	0·00725	138	13	0·01763	57
5	0·01130	88	15	0·01824	55
7	0·01244	80	17	0·01814	55
9	0·01471	68	19	0·01930	52

*The formaldehyde release is expressed in mol.prop./glucose residue.

These results are shown graphically in figure 11, facing p.74. The experiment was repeated but the astasian was shaken for 5-10 min. with 1N-NaOH (6 ml.) and neutralised before addition of periodate and diluting to 25 ml. The sampling proved difficult because of the gelatinous nature of the suspension. The results did, however, indicate that no appreciable degradation occurred during the very brief pre-treatment with alkali.

Overoxidation of Astasian.

Astasian (10·1 mg.) was oxidised with 0·3M-sodium meta-periodate (2 ml.) in phosphate buffer, pH 8·0 (12·5 ml.) in at total volume of 25 ml. at 37°. The mixture was continuously stirred in the dark and samples (1 ml.) taken for estimation of formaldehyde. The release of formaldehyde was 0·60 mol.prop./ glucose residue in one experiment and 0·65 mol.prop. in another.

Consumption of Periodate by Astasian.

The periodate consumption was measured by the spectrophotometric method of Aspinall and Ferrier (1957). Astasian (70·1 mg.) was oxidised with 0·015M-sodium metaperiodate (10 ml.) at 27° for 72 hr. The mixture was shaken and samples (2 ml.) centrifuged. A portion of the supernatant solution (1 ml.) was diluted to 250 ml. and the absorbance measured against a water blank at 222·5 mμ using a Unicam S.P.500 Spectrophotometer. The original 0·015M-sodium metaperiodate solution was diluted 250 times and read against distilled water. An equimolecular solution of sodium iodate was prepared by treating a sample (1 ml.) of the periodate

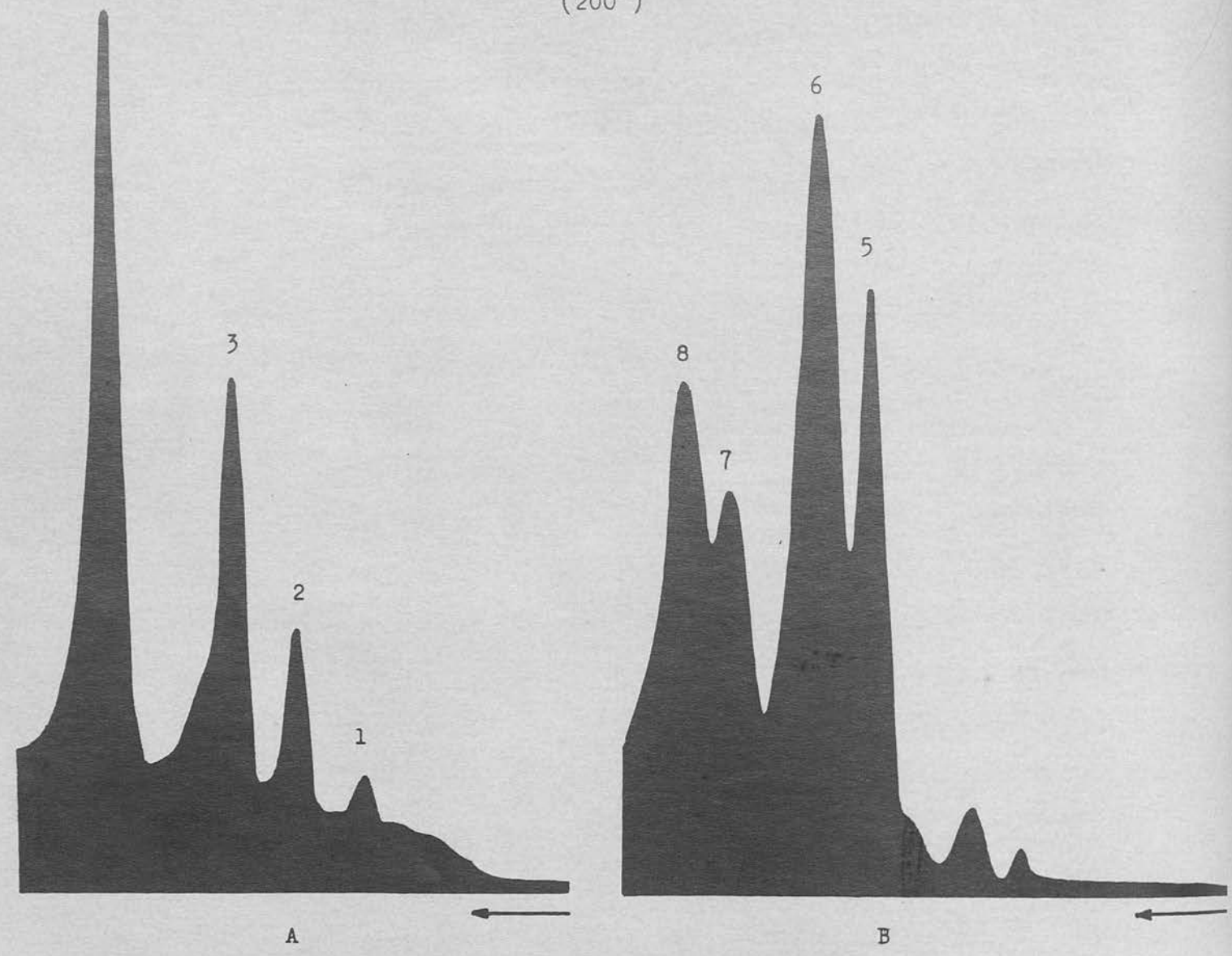
solution with 2 drops of ethylene glycol for 15 min. at room temperature, followed by dilution to 250 ml. The spectrophotometer readings were; periodate, 0·601; iodate, 0·085; 72 hr. oxidation sample, 0·390. These results correspond to a periodate consumption of 0·14 mol.prop./glucose residue. In identical experiments with laminarin and peraneman Cunningham (1961) found values of 0·30 and 0·03 mol.prop. respectively.

Methylation of Astasian.

Astasian (3 g.) dissolved in dimethyl sulphoxide (50 ml.) and dimethyl formamide (50 mg.) was methylated with dimethyl sulphate (35 ml.) in the presence of barium oxide (17 g.) and barium hydroxide octohydrate (17 g.). The reaction was carried out according to the method of Kuhn and Trischmann (1963) and yielded only 0.4 g. of undermethylated product. The methylation was continued by three silver oxide, methyl iodide, dimethyl formamide treatments according to the conditions of Kuhn, Trischmann and Löw (1955). The yield from these experiments was too low to allow a complete analysis to be carried out. The reason for the low yield might be either due to degradation of the polysaccharide or to the conditions of Kuhn and Trischmann (1963) being unsuitable for the methylation of astasian or a combination of both. The product was therefore examined only by chromatographic methods.

GAS CHROMATOGRAMS OF METHYL GLYCOSIDES FROM METHYLATED ASTASIA GLUCAN

Column: polyphenyl ether [m-bis-(m-phenoxyphenoxy)-benzene]. (200°)



1 and 2 correspond to the methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucose.

3 and 4 correspond to the methyl glycosides of 2,4,6-tri-O-methyl-D-glucose.

5 and 7 correspond to the methyl glycosides of 2,4-di-O-methyl-D-glucose.

6 and 8 do not correspond to the methyl glycosides of the 2,3- or 3,4-isomers.

FIGURE 17

Total Acid Hydrolysis of Methylated Astasian.

A few milligrams of the methylated product were heated in a sealed tube with 90% formic acid (0.1 ml.) at 100° for 1 hr., followed by removal of the formic acid by successive evaporations with water. 2N-H_2SO_4 (0·5 ml.) was added and the solution heated at 100° for 2 hr., followed by neutralisation with barium carbonate. Using 4:1:5 and 200:17:1 as chromatographic solvents, spots corresponding to authentic samples of 2,3,4,6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-glucose were detected.

Methanolysis of Methylated Astasian.

A few milligrams of the product were heated with 3% methanolic hydrogen chloride at 100° for 18 hr. in a sealed tube. The hydrogen chloride was removed by successive evaporations with anhydrous methanol. The gas-liquid chromatographic analysis of the product (performed by Dr. G.O. Aspinall) confirmed the presence of 2,3,4,6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-glucose as their methyl ethers (see Fig.17A) thus providing further evidence for the presence of (1→3)-glucosidic linkages.

Reduction of Astasian to Astasitol.

The direct reduction of the insoluble astasian granules gave a product, which on periodate oxidation yielded less formaldehyde than was expected. (The degree of polymerisation on the basis of two molecules of formaldehyde per sorbitol end group was 75-80). This indicated that reduction was incomplete and experiments were

carried out to achieve reduction of the polysaccharide in solution.

In a preliminary experiment, maltose (200 mg.) was dissolved in dimethyl sulphoxide (20 ml.) and potassium borohydride (100 mg.) in distilled water (0.5 ml.) added. The solution was allowed to stand overnight, neutralised with acetic acid and the dimethyl sulphoxide removed. The residual syrup was dissolved in water, deionised and hydrolysed with acid. Chromatographic examination of the hydrolysate indicated the presence of glucose and sorbitol in approximately equal amounts.

Astasian (3.3 g.) was dissolved in dimethyl sulphoxide (400 ml.) and a solution containing potassium borohydride (1 g.) in water (30 ml.) added very slowly with vigorous stirring. The mixture remained as a clear, homogeneous solution for 24 hr., but thereafter became slightly opaque and gel-like. After 27 hr. the mixture had become very viscous. The gel was stirred with ethanol for 1 hr. and the voluminous precipitate centrifuged down. The precipitate was washed very thoroughly with ethanol and ether before drying. (Preliminary experiments showed that this was essential, to avoid caking of the precipitate on drying). The polysaccharide was dissolved in dimethyl sulphoxide (300 ml.) by warming for 2-3 hr. and potassium borohydride (1 g.) dissolved in water (20 ml.) added. The solution behaved exactly as described above. The product was worked up as previously described and the process repeated. After this third reduction the gel was not dried but merely centrifuged free from dimethyl sulphoxide as far as possible. It was then suspended in water (200 ml.) and potassium borohydride

(2 g.) added slowly, with stirring. The mixture was stirred as a gel-like slurry for 12 hr. 5N-NaOH was added at a rate of 1 drop every 5 min., in an atmosphere of nitrogen, and, when the normality of alkali in the mixture was about 0·1N, a clear solution was formed. The solution was stirred under nitrogen at room temperature for 48 hr. The solution was neutralised with acetic acid to pH7 and an equal volume of ethanol added. The precipitate was centrifuged down and washed thoroughly with 50% ethanol (300 ml. x 6), ethanol (100 ml. x 4), butanol (100 ml. x 4) and ether (100 ml. x 4). The solid material was dried at 60° over phosphorus pentoxide *in vacuo*, ground up in an agate mortar, and re-dried to constant weight. The yield of astasitol was 3·05 g.

Examination of Astasitol.

The product exhibited the same solubility properties as astasian, but the particles of the solid material were very hard and did not form a suspension in water. Pretreatment with alkali was therefore necessary before periodate oxidation.

Astasitol (45·0 mg.) was dissolved in 1N-NaOH (8 ml.). Portions (0·2 ml.) were hydrolysed successively with 90% formic acid (1 ml.) and 2N-H_2SO_4 (2 ml.) and neutralised, prior to estimation of glucose. Glucose (5·84 mg./ml.) was found, corresponding to a polyglucose content of 93·5%. The alkaline solution (7 ml.) was neutralised and oxidised with 0·3M-sodium metaperiodate (2 ml.) in a total volume of 15 ml. Since the sampling of the

gelatinous suspension was uncertain portions (1 ml.) were withdrawn for estimation of formaldehyde only when the reaction was approaching completion. The production of formaldehyde after 13, 16 and 19 days was 0.0364, 0.0352 and 0·0394 mol.prop./glucose residue respectively, confirming the general order of magnitude of the degree of polymerisation obtained by periodate oxidation of astasian (i.e. 50-55 glucose residues).

Methylation of Astasitol

The methylation was carried out by the method of Kuhn and Trischmann (1963) as modified by Dr. D. A. Rees (personal communication). Astasitol (2·75 g.) was dissolved by stirring in dimethyl sulphoxide (200 ml.) at 60° for 3 hr. in an atmosphere of nitrogen. The solution was cooled to 2° and dimethyl formamide (200 ml.) and barium hydroxide octahydrate (200 g.) added. The mixture was stirred vigorously at 2° for 30 min. to produce a thick white slurry and the first portion of dimethyl sulphate (36 ml.) added. Subsequent additions of dimethyl sulphate (36 ml. each) were made 1 hr., 1·5 hr. and 2 hr. after the first addition, making the total volume of dimethyl sulphate added 144 ml. The ice bath was removed 30 min. after the last addition and the mixture allowed to come to room temperature. After about 30-40 min. the reaction mixture began to heat up and the temperature rose to about 40-45°, producing an almost clear solution. The apparatus was sealed off with a calcium chloride tube to prevent

the mixture from picking up moisture and gentle stirring continued at room temperature for 3 days. The mixture was then stirred with 0.88-ammonia (60 ml.) for 1 hr. to destroy the excess dimethyl sulphate. Chloroform (300 ml.) was added, the mixture warmed under reflux until the chloroform boiled and then the mixture stirred vigorously. The upper chloroform layer was separated off after centrifuging the mixture. This procedure was repeated four times using 100 ml. quantities of chloroform. The combined chloroform extracts were washed six times with distilled water (200 ml.). During this procedure, gels were formed, which could be separated into an aqueous and a chloroform layer by centrifugation at 1,500 r.p.m. for 15-20 min. The chloroform was removed using a rotary-film evaporator, but removal of the remaining dimethyl formamide necessitated the use of a high vacuum pump. 1.70 g. of partly methylated product was obtained corresponding to a yield of 62% (w/w).

The partly methylated product was insoluble in methyl iodide but dissolved readily in dimethyl formamide. The procedure of Kuhn, Trischman and Löw (1955) was therefore adopted. The partly methylated product (1.70 g.) was dissolved in dimethyl formamide (20 ml.) and methyl iodide (20 ml.) and silver oxide (6 g.) added. The mixture was stirred in the dark at room temperature for 24 hr. before filtering off the silver oxide. The silver oxide was washed thoroughly with hot chloroform and the combined filtrates evaporated to dryness. A total of three such methylations were

performed. A few milligrams of the product were hydrolysed and the neutralised hydrolysate examined by paper chromatography. Traces of monomethyl sugars were detected indicating that the product was not fully methylated.

The material readily dissolved in methyl iodide (30 ml.) and the solution was refluxed with silver oxide (6 g.) for 48 hr. The silver oxide was removed by filtration and washed thoroughly with hot chloroform. The combined filtrate and washings were evaporated to dryness and the process repeated. The methylated product weighed 0·95 g. and had a methoxyl content of 43·9%, The methoxyl content was found by the Ziesel method and was analysed by Mr. M. Fleming.

Total Hydrolysis of Methylated Astasitol and Separation of the Methylated Sugars.

The product (0·90 g.) was heated with 90% formic acid (20 ml.) for 1 hr. at 100° and the formic acid removed by successive evaporations with water. 2N-H_2SO_4 (20 ml.) was added and heating continued for 2 hr. Neutralisation was carried out using barium carbonate.

A cellulose column was prepared by boiling a slurry containing Whatman standard grade cellulose with a solution of methyl ethyl ketone/water, 10:1 (v/v). The hot slurry was poured into a column (3.5 cm. diam.) and packed tightly by mechanical pressure and by passing several litres of the solvent through it under compressed air, to give a final height of about 40-45 cm. The column was

fitted with a fixed bend of glass capillary tubing from the outlet to give a slow flow rate.

A portion of the methylated astasitol hydrolysate was kept back for determination of the ratio of methylated sugars, and the rest was applied to the column. The column was eluted with methyl ethyl ketone/water, 10:1 (v/v) at a rate of 11 ml./hr. and fractions (5 ml.) collected. The column fractions were analysed by paper chromatography using 4:1:5 as solvent and aniline oxalate spray reagent.

Fractions 36-40 contained tetramethylglucose (R_F 0·81), 42 contained tetramethylglucose and a trace of trimethylglucose (R_F 0·65), 44-46 contained trimethylglucose (R_F 0·65) and a trace of another sugar (R_F 0·73), fractions 48-68 contained only trimethylglucose (R_F 0·65) and fractions 72-154 probably contained dimethyl sugars.

Fractions 36-40 were evaporated to dryness to yield 13 mg. of a syrup which crystallised over a period of 6 days. After three recrystallisations from light petroleum (b.p. 40-60°) the m.p. and mixed m.p. with an authentic sample of 2,3,4,6-tetra-O-methyl-D-glucose was 84-85°. The material ran as a single spot, with an R_F value identical to the authentic sample of tetramethyl-glucose, on paper chromatography with 200:17:1 and 4:1:5 as solvents. The specific rotation of the crystals was $[\alpha]_D^{17}$ +85° (c, 0·6 in H_2O after 12 hr.) and the X-ray powder photograph of the crystals was identical to that of the authentic sample of 2,3,4,6-tetra-O-methyl-D-glucose.

Fractions 48-68 crystallised immediately on evaporation to dryness (yield 700 mg.). The crystals ran as a single spot when examined by paper chromatography using 200:17:1 and 4:1:5 as solvents and the mobility and pink colour with aniline oxalate corresponded exactly with those of an authentic sample of 2,4,6-tri-O-methyl-D-glucose. The 2,3,6-isomer had an R-tetramethyl-glucose-value of 0·63 (cf. 0·55 for the 2,4,6-isomer), using 200:17:1 as solvent and gave a brown colour with aniline oxalate. After three recrystallisations from ethyl acetate the crystals melted at 123-124° (undepressed on mixing with an authentic sample of 2,4,6-tri-O-methyl-D-glucose). The X-ray powder photograph was identical with the authentic sample of the 2,4,6-isomer and differed from the 2,3,6-isomer.

Fractions 43-47 on evaporation yielded a syrup (ca. 2 mg.). On paper chromatography, the major spot corresponded to 2,4,6-tri-O-methyl-D-glucose. The minor component, which comprised only about one tenth of the mixture (visual estimation) corresponded to an authentic sample of 2,3,4-tri-O-methyl-D-glucose. Since the amount of this sugar was so minute, it is not thought that it is of structural significance.

Fractions 72-154 on evaporation to dryness yielded 14 mg. of a syrup which did not crystallise. It was methanolised by heating 4-5 mg. of the syrup with 3% methanolic hydrogen chloride (0·5 ml.) in a sealed tube at 100° for 5 hr. The hydrogen chloride was removed by repeated evaporation with dry methanol and the methanolised product examined by gas-liquid chromatography

(performed by Dr. G.O. Aspinall). The results are shown in figure 17, facing p.103. Evidence for the presence of 2,4-di-O-methyl-D-glucose is indicated but the identity of peaks 6 and 8 is not certain. These peaks do not correspond to the 2,3- or the 3,4-isomers but could be due to the presence of the α- and β-methyl glycosides of 4,6-di-O-methyl-D-glucose. Since at least two dimethyl derivatives are present it is difficult to assess any structural significance of the dimethyl fraction.

Ratio of Methylated Sugars.

The ratio of methylated sugars was estimated from a portion of the hydrolysate of methylated astasitol, using the method of Schaefer and Van Cleve (1956) which is described in chapter 1, section B. Calibrations were carried out using authentic samples of 2,3,4,6-tetra-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-glucose and 4,6-di-O-methyl-D-glucose. This latter sugar was chosen since gas chromatographic evidence indicated that it was probably the major component of the dimethylglucose fraction. The calibration graphs are shown in figure 9 facing p. 68. The results gave a molar ratio of tetramethyl:trimethyl:dimethyl of 2·22:95·34:2·44 in one experiment, and 2·40:94·74:2·86 in a second experiment. These results indicate an average chain length of about 45. In parallel experiments with hydrolysates of methylated laminaritol (from laminarin sample BB2) and methylated astasian (see p. 102) the chain length value in each case was in the region of 20-25 glucose residues, confirming that severe degradation of astasian had taken place during methylation.

Discussion

Cultures of *Astasia ocellata* were grown by Dr. J.F. Ryley and the cells stored in methanol. The cells were disrupted by ultrasonic vibrations and the bulk of the protein material removed by digestion with trypsin followed by extraction with urea solution to remove the amino acids and protein fragments liberated (Clarke and Stone 1960). Further removal of denatured protein was attained by differential centrifugation and the final traces removed by shaking a saline suspension of the material with toluene (cf. Cowie and Greenwood, 1957). The pure white granules of paramylon were obtained in 55% yield (on a dry cell basis). The granules (polyglucose content 96%) did not stain with iodine and were insoluble in boiling water and in dilute mineral acids; they were, however, soluble in N-NaOH and in dimethyl sulphoxide. On total acid hydrolysis the granules gave only glucose, the presence of which was verified by its complete conversion to gluconic acid when treated with a D-glucose oxidase preparation; and on partial acid hydrolysis a homologous series of oligosaccharides was produced with R_G -values corresponding to the laminarisaccharides, suggesting the presence of β-(1→3)-linkages. The low specific rotation $[\alpha]_D^{18}$ +17°, c 1·02 N-NaOH; the infrared spectrum with a characteristic peak at 890 $cm.^{-1}$ (see figure12 facing p. 78) and the action of an exo-β-glucosidase preparation from *Rhizopus arrhizua* confirmed the presence of β-glucosidic linkages. The insolubility of astasian prevented a quantitative

study of the action of the Rhizopus arrhizus preparation, but chromatographic examination of the enzyme digest indicated the presence of glucose, laminaribiose and laminaritriose. Analogous experiments with other β-(1→3) glucans (paramylon from Euglena gracilis, "insoluble"laminarin and a sample of chrysolaminarin, provided by Dr. E.E. Percival) produced the same three sugars.

The polysaccharide was resistant to borohydride reduction; a single reduction in aqueous suspension with potassium borohydride converted only about one quarter of the reducing end-groups to sorbitol residues. A method was therefore devised for the reduction of the granules in solution. The reduction was achieved by careful addition of an aqueous potassium borohydride solution to a solution of the granules in dimethyl sulphoxide. The process was repeated and a final reduction carried out in aqueous alkali. The reduced polysaccharide, which precipitated on neutralisation was very gelatinous and successive washings with ethanol, butanol and ether were necessary before the material could be dried without caking. The product had a polyglucose content of 93·5% and the recovery of polysaccharide material was 86%.

Periodate oxidation experiments were performed on astasian and on the corresponding alcohol astasitol. The formaldehyde produced on oxidation of astasian with sodium metaperiodate at room temperature (0·018-0·019 mole prop./glucose residue) corresponded to an average degree of polymerisation of 50-55 glucose

residues, assuming that one molecule of formaldehyde is liberated from each polysaccharide molecule (see figure 14 facing p.83.) Oxidation of astasian at 2° yielded no significant quantities of formaldehyde even on prolonged oxidation, confirming the absence of sugar alcohol terminated molecules such as are present in laminarin (Anderson et al., 1958). Technical difficulties were encountered in periodate experiments on astasitol. The particles of the dried material were very hard and dense and sampling could not be carried out with certainty. (Sampling of suspensions of the unreduced polysaccharide was relatively simple, since the granules took several minutes to settle under gravity). The astasitol was therefore dissolved in alkali and the concentration of polysaccharide determined by estimating the glucose liberated on total acid hydrolysis. The alkaline solution was neutralised and periodate oxidation carried out. The gelatinous nature of the suspension made sampling difficult. The results did, however, confirm that the average degree of polymerisation was 50-55 glucose residues. The low periodate consumption (0·14 mol.prop./glucose residue), being intermediate between that of laminarin and of peraneman, provided further evidence for (1→3)-linkages. The yield of formaldehyde on "overoxidation" of astasan was about 0·6 mol.prop./glucose residue. The reason for the incomplete over-oxidation is not known.

Preliminary methylation studies on astasian gave a product, which, on acid hydrolysis and examination by paper chromatography, yielded spots corresponding to authentic samples of 2,3,4,6-tetra-

O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-glucose. The latter compound was quite different from an authentic sample of the 2,3,6-isomer both in its colour reaction with aniline oxalate and in its chromatographic mobility. Confirmation of the presence of 2,3,4,6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-glucose was obtained by methanolysis of a sample of the product and examination of the mixed glycosides using a Pye Argon Chromatograph and two different columns (Aspinall, 1963). (The gas chromatograms were run by Dr. G.O. Aspinall). The low yield of product and the relatively high proportion of tetramethylglucose did, however, indicate that degradation had occurred during methylation.

A complete methylation study was therefore carried out on the borohydride-reduced polysaccharide. Complete methylation of substantially linear β-(1→3)-glucans is generally difficult (see for example Perlin and Taber, 1963). A total of six successive methylations was performed, comprising one barium hydroxide-dimethyl sulphate in dimethyl formamide and dimethyl sulphoxide methylation (Kuhn and Trischmann, 1963; as modified by Dr. D. A. Rees, personal communication); three methyl iodide-silver oxide in dimethyl formamide, methylations (Kuhn, Trischmann and Löw, 1955) and two methyl iodide-silver oxide methylations (Purdie, 1903). The product (methoxyl content, 43·9%) was hydrolysed successively with formic acid and sulphuric acid and neutralised. The methylated sugars were separated on a cellulose column. The 2,3,4,6-tetra-O-methyl-D-glucose (13 mg.) was characterised by its m.p.

and mixed m.p. with an authentic sample (84-85°), specific rotation $[\alpha]_D^{17}$ +85° (c 0.6, water) and X-ray powder photograph. The 2,4,6-tri-O-methyl-D-glucose (700 mg.) was characterised by its chromatographic mobility, its m.p. and mixed m.p. (123-124°) and by an X-ray powder photograph.

A fraction intermediate between the tetramethyl and the main trimethyl fractions yielded a syrup (2 mg.). On paper chromatography the major spot corresponded to 2,4,6-tri-O-methyl-D-glucose and a minor component which comprised only about one tenth of the mixture corresponded to an authentic sample of 2,3,4-tri-O-methyl-D-glucose. Since the amount of this latter sugar is so small, it is difficult to assign to it any structural significance and it is probably an artefact of the methylation procedure.

The dimethyl fraction was methanolysed and the mixed glycosides examined by gas-liquid chromatography (figure 17B). The presence of the glycosides of at least two dimethyl sugars was indicated, suggesting that they may have arisen from under-methylation or from demethylation during hydrolysis.

The ratio of methylated sugars (determined by the method of Schaefer and Van Cleve, 1956) was tetramethyl:trimethyl:dimethyl 2·31:95·04:2·65 indicating an average chain length of about 45 glucose units. This value together with the value of about 50 glucose units for the average degree of polymerisation obtained from periodate studies indicates that the molecule is substantially linear. This is in agreement with the complete insolubility of the molecule in water. The relationship between solubility

properties and degree of branching has already been mentioned in the discussion on leucosin (see p. 86); perhaps an even more striking example of this is the glucan from Fungi imperfecti (Johnson et al., 1963). The basic structure of the water-soluble glucan is shown in figure 13 (b), facing p. 82.

When the glucan is successively periodate oxidised, reduced and partially hydrolysed the branch "stubs" are removed and a linear product (c) results. The degraded glucan closely resembles the paramylon granules from Astasia ocellata and Peranema trichophorum both in its lack of solubility in water and in its resistance to periodate oxidation. A comparison of the solubility properties of some glucans is given below.

Source of glucan	Degree of polymerisation (glucose residues)	Solubility in water	Solubility in alkali
"Smith" degraded glucan from Fungi imperfecti	85 (linear)	insoluble	insoluble
Peramena Trichophorum	80 (linear?)	insoluble	partly dissolves after 5 hr. in 2N-NaOH.
Astasia ocellata	50 (substantially) linear)	insoluble	readily soluble in N-NaOH
Ochromonas malhamensis	35 (branched)	soluble	soluble

It is therefore concluded that the reserve polysaccharide from Astasia ocellata is an essentially linear polymer of D-glucose units linked by β-(1→3)-glucosidic bonds. The molecular structure of the polysaccharide appears to differ from the reserve polysaccharide of

Peranema trichophorum only in its degree of polymerisation. The present studies provide the first methylation analysis of protozoal paramylon granules.

Table 3. Properties of some β-(1→3)-glucans

Property	Laminarin	Chrysolaminarin (1)	Leucosin	Astasian	Peraneman	Euglena gracilis paramylon(2)
$[\alpha]_D$ (water)(°)	-9	-6	+10	.	.	.
$[\alpha]_D$ (NaOH)(°)	+9	.	.	+17	+16	+28
Infrared-absorption spectrum (absorption peak) ($cm.^{-1}$)	890	890	890	890	890	890
Periodate oxidation at pH 5, reduction (molecule/anhydro-glucose residue)	0.30	0.30	0.17	0.14	0.03	0.02
Average degree of polymerisation (glucose residues)	20	21	36	50-55	80	150
Hydrolysis by Rhizopus preparation	+	+	+	+	+	+

(1) See Beattie et al. (1961). (2) See Clarke and Stone (1960).

CHAPTER 3

STUDIES ON THE TRANSFERASE ACTIVITY OF A CELL-FREE EXTRACT FROM TETRAHYMENA PYRIFORMIS

INTRODUCTION.

Tetrahymena pyriformis is a free living ciliate commonly found in fresh water ponds. The organism can readily be grown in bacteria-free culture and it has a high rate of growth and reproduction. The metabolism of the organism has therefore been extensively investigated (for reviews, see Corliss, 1954 and Koehler and Fennel, 1964). Manners and Ryley (1952) showed that Tetrahymena pyriformis synthesises a reserve polysaccharide which closely resembles animal glycogen. The enzyme systems responsible for the synthesis of the reserve polysaccharide have been investigated by Ryley (1952). Cell-free extracts of the organism were found to possess succinic and lactic dehydrogenases, aldolase, hexokinase, oxoisomerase, phosphoglucomutase and phosphorylase activities; amylase and maltase activities were also detected. Archibald and Manners (1959) investigated the trans α-glucosylase activity and showed that by using concentrated solutions of maltose, oligosaccharides were synthesised. Since the Tetrahymena pyriformis extract was unable to polymerise glucose it was concluded that the oligosaccharides were formed by transglucosylation. Examination of the oligosaccharides showed that the hydroxyl groups at both C-4 and C-6 of the non-reducing residue of maltose and maltotriose can function as acceptors for α-glucosyl radicals. The isolation of isomaltose indicated that C-6 of glucose also acts as an

acceptor site.

Maltose as Donor and Acceptor Substrate.

In general, maltases acting on maltose as both substrate and acceptor produce either maltotriose (if transfer is to the terminal C-4), or panose (if transfer is to the terminal C-6), and isomaltose, if C-6 of the liberated glucose is also able to act as an acceptor site. The transfer of α-glucosyl groups from maltose to hydroxyl groups at C-2 and C-3 of glucose does not appear to be common, although the synthesis of nigerose by an Aspergillus oryzae preparation has been reported (Pazur, Budovich and Tipton, 1957) and more recently Lukomskaya (1963) has isolated kojibiose from a digest containing a rabbit liver enzyme preparation and maltose. Apart from these apparently unusual examples, maltose-hydrolysing systems can be classified into three types involving transfer to C-4 or C-6, or both. Thus several fungal maltases transfer only to C-6 of both glucose and maltose (see for example Pazur and French, 1952); whereas maltases from Phaseolus radiatus (Nigam and Giri, 1960) and from the rumen protozoan Entodinium caudatum (Bailey and Howard, 1963) transfer only to C-4 of maltose. The maltases from Cladophora rupestris (Duncan and Manners, 1958) and from Dasytricha ruminantium (Bailey and Howard, 1963) can transfer to both C-4 and C-6 of maltose but not to C-6 of glucose. The enzyme system in Tetrahymena pyriformis appears to fall into this latter group except that it can also transfer glucosyl radicals to C-6 of glucose.

Cellobiose as Donor and Acceptor Substrate.

In general trans β-glucosylases appear to show a lower specificity towards the acceptor molecule than the corresponding trans α-glucosylases, although transfer to a primary alcohol group may predominate. Differences in acceptor specificity between enzyme preparations from different biological sources are not so marked (for reviews of the recent literature see Manners, 1960 and Hutson, 1964). Using cellobiose as donor and acceptor substrate the main transfer products are generally gentiobiose, 6^2-β-glucosyl-cellobiose, cellotriose and gentiotriose. Laminaribiose and sophorose and higher oligosaccharides containing (1→3) or (1→2)-linkages may also be formed. The formation of these and other transfer products may vary depending on (a) the concentration of cellobiose and (b) the extent to which the reaction is allowed to proceed (see Figure 21).

Transfer of Glucosyl Radicals to Acceptors Other Than Glucose.

The transfer of glucosyl radicals to various acceptors such as alcohols or pentoses may provide further information on the acceptor specificity of the enzyme system and may also lead to the synthesis of compounds which would be difficult to prepare by chemical means. For example, using cellobiose as glucosyl donor and xylose as acceptor 3-O-β-D-glucopyranosyl-D-xylose has been synthesised using enzyme extracts from Cladophora rupestris (Duncan, Manners and Thompson, 1959) and from Aspergillus niger (Barker, Bourne, Hewitt and Stacey, 1957). Using a yeast preparation Avigad (1959) prepared maltulose from a mixture of maltose and fructose.

Conditions for Transfer.

It has been suggested that all hydrolytic enzymes capable of attacking more than one substrate may be expected to act as transglycosylases under suitable conditions (Morton, 1953). Thus a highly specific trehalase preparation from hog intestines cannot catalyse the transfer of glucosyl radicals to acceptors other than water (Dahlqvist, 1960), whereas most maltases generally hydrolyse more than one substrate, and, at high concentrations of maltose, transfer occurs. Some of the glucosyl-transferring enzymes do not produce appreciable hydrolysis i.e. water is not the most favourable acceptor. For example, D.J. Manners and D.C. Taylor (unpublished results) have shown that an enzyme preparation from the flagellated protozoan Astasia occelata possesses laminaribiose phosphorylase activity. Little or no hydrolysis of α-glucosyl phosphate occurs but, in the presence of added glucose, laminaribiose is formed:

α-glucosyl phosphate + glucose $\rightleftharpoons$ 3-O-β-glucosylglucose + inorganic phosphate

On the other hand, several enzymes catalyse both hydrolysis and transfer to a variable degree. Thus according to Hassid and Neufeld, 1963, there is a continuous spectrum of glucosyl-transferring enzymes ranging from those with exclusive hydrolytic activity to others with no hydrolytic activity at all.

Mechanism of Transglucosylation Reactions.

Transglucosylation reactions can be represented by the general equation:

$$G\text{-}O\text{-}R + H\text{-}O\text{-}X \rightleftharpoons G\text{-}O\text{-}X + H\text{-}O\text{-}R$$

In most transglucosylation reactions, the configuration of the glucosidic linkage is retained. The mechanism may therefore be:

$$G\text{-}O\text{-}R + Enz\text{-}H \rightleftharpoons G\text{-}Enz + H\text{-}O\text{-}R$$

$$G\text{-}Enz + H\text{-}O\text{-}X \rightleftharpoons G\text{-}O\text{-}X + Enz\text{-}H.$$

Koshland (1959) suggested that the above reactions involve two successive Walden inversions, although direct evidence for the formation of an intermediary β-glucosyl-enzyme complex during trans-α-glucosylation is not yet available.

A few reactions involving inversion of configuration have been reported. For example Putnam, Litt and Hassid (1955) showed that an enzyme preparation from Neisseria meningitidis catalyses the transfer of glucosyl radicals from β-glucosyl phosphate to xylose, resulting in the formation of 4-O-α-D-glucopyranosyl-D-xylopyranose. Such reactions may occur by single displacement mechanisms (Koshland, 1959).

Scope of the Present Investigation.

In the present investigation studies on the specificity of the trans-α-glucosylase activity of a cell-free extract from Tetrahymena pyriformis have been continued with particular reference to the

products present at equilibrium. Transfer of glucosyl radicals from phenyl α-glucoside to various acceptors has been investigated and several new disaccharides synthesised. Preliminary studies on the trans-β-glucosylase activity of the extract have also been carried out.

EXPERIMENTAL METHODS.

1. Drying of Disaccharide Samples.

With samples which did not crystallise, the syrup was evaporated to dryness successively with ethanol and a mixture of ethanol and acetone at 40° until an amorphous solid was produced. The solid material was ground down to a fine powder and dried to constant weight under vacuum at 60° over P_2O_5. With most samples "frothing" occurred at this temperature, indicating removal of moisture, but in a few cases a brief treatment at 100° was necessary for complete removal of moisture.

2. Acid Hydrolysis.

Disaccharides (2 mg.) were heated at 100° with 1.5N-H_2SO_4 (0.2 ml.) for 2 hr. Barium carbonate was used for neutralisation.

3. Reduction of Disaccharides.

The disaccharide (2 mg.) was treated with potassium borohydride (2 mg.) in water (1 ml.) for 24 hr. at room temperature and the resulting solution deionised with Amberlite IR-120(H) resin. Borate ions were removed by successive evaporations with methanol.

4. Paper Chromatography.

Paper chromatograms were developed in the solvents described in chapter 2, section B. In addition to the aniline oxalate and silver nitrate reagents described in chapter 1, section B, the following reagents were used as spot-tests and as chromatographic

sprays:-

(a) The periodate-permanganate reagent (Lemieux and Bauer, 1954) was used for detection of sugar alcohols on chromatograms developed in 9:1:1.

(b) Triphenyl tetrazolium chloride spray reagent (Wallenfels, 1950) was used as a specific test for (1→2)-linked disaccharides.

(c) Aniline-diphenylamine spray reagent was used to indicate reducing sugars and, specifically, 4-O-substituted reducing end groups of oligosaccharides, which gave a blue coloration (Schwimmer and Bevenue, 1956).

(d) Periodate-p-rosaniline spray reagent (Hardy and Buchanan, 1963) was used, with the following modification. Disaccharides were sprayed with a solution containing equal volumes of a 2% sodium metaperiodate solution and 0.1 M-phosphate buffer (pH 8.0). The chromatogram was left at room temperature for 5 min. before treatment with sulphur dioxide and spraying with the p-rosaniline reagent. The inclusion of phosphate buffer gave clearer yellow colours with 3-O-substituted hexoses and pentoses and in several cases the blue centres to spots were avoided completely. Reduced disaccharides were treated exactly as described by Hardy and Buchanan (1963). The uses of

this spray reagent are discussed on p.175.

5. Paper Electrophoresis.

All electrophoretograms were developed using a Shandon small scale electrophoresis apparatus, using 10-20 volt./cm. for 0.5-2.5 hr. Reducing di- and trisaccharides were run in borate buffer (pH 10.0) (Foster, 1953). The best results were obtained using 0.05 M-borate.

Reduced compounds were run in molybdate buffer (Bourne, Hutson and Weigel, 1961). Using the conditions described by these authors and the Shandon (non-cooled) electrophoresis apparatus dark backgrounds were produced with the silver nitrate spray reagent. By reducing the concentration of buffer by half, this was avoided. The buffer was prepared by dissolving sodium molybdate (12.5 g.) in water (1,200 ml.) and adjusting the pH to 5.0 with sulphuric acid.

6. Methylation.

Methylation of di- and trisaccharides was carried out according to the procedure of Kuhn, Trischmann and Löw (1955). The sugar (0.5 - 2.0 mg.) was shaken with methyl iodide (0.2 ml.), dimethyl formamide (0.2 ml.) and silver oxide (0.2 g.) in the dark at room temperature for 24 hr. Two such treatments were generally sufficient.

The product was divided into two portions; the first portion was hydrolysed with 2N-H_2SO_4 for 2 hr. at 100°, followed by neutralisation with barium carbonate and examination by paper

chromatography using 200:17:1 as solvent and aniline oxalate spray reagent. The second portion was treated with 3% methanolic hydrogen chloride for 5-6 hr. The hydrogen chloride was removed by repeated evaporation with methanol and the product examined by gas-liquid chromatography using a Pye Argon Chromatograph. The analyses of the products from the cellobiose transfer digest were carried out by the author; all others were kindly performed by Dr. G.O. Aspinall.

7. Preparation of Compounds.

(a) Phenyl-α-D-glucopyranoside was prepared from α-pentacetyl-glucose, by treatment with phenol and zinc chloride in acetic acid and acetic anhydride (Montgomery, Richtmyer and Hudson, 1942). The tetracetyl phenyl-α-glucoside so formed was recrystallised six times from ethanol before deacetylation by the method of Isbell (1930). The product had $[\alpha]_D^{16}$ + 186°.

(b) 3-O-α-D-glucopyranosyl-D-arabinose was prepared from maltose by the method of Whistler and Yagi (1961).

(c) 5-O-α-D-glucopyranosyl-D-arabinofuranoside was prepared isomaltose by the method of Whistler and Yagi (1961).

8. Periodate Oxidation.

The reduction of periodate was followed by the spectrophotometric method of Aspinall and Ferrier (1957).

The formaldehyde released during oxidations was determined by the chromotropic acid method (MacFadyen, Watkins and Anderson, 1945).

Estimation of Glucose.

Glucose was determined specifically using a glucose oxidase-peroxidase-o-toluidine system as described by White and Subers (1961). The reagent showed no α- or β-glucosidase activities.

EXPERIMENTAL.

1. Preparation of Extracts. (by Dr. J.F. Ryley).

The work in this chapter was carried out using two batches of extracts, prepared by Dr. J.F. Ryley. The first extract (hereinafter referred to as extract I) was less active than the second (extract 2). Extract I was the same as that used by Archibald and Manners (1959) but was generally similar to extract 2 which was prepared as follows:

thirty one 2 l.conicals, each containing 500 ml. medium (1% peptone, 0.4% sodium chloride) inoculated with one tube each of a 5 day culture and incubated in the dark at 24°. The cells were harvested after 7 days, washed once in 0.4% sodium chloride and once in citrate buffer of pH 6.0. The cell suspension was diluted to 200 ml. with 0.05M-citrate buffer of pH 6.0 and freeze dried.

2. Sugar Impurities in the Extracts.

When the extracts (10 mg.) were dissolved in water (1 ml.) and the solution examined by paper chromatography using 10:4:3, 18:3:1:4 and 4:1:5 as solvents a small amount of glucose and a minute trace of a spot, with an R_G-value identical to that of D-ribose, were detected. The impurities were further examined by dialysing the extract and concentrating the diffusible material to small volume. The solution was deionised with Amberlite IR-120(H) and IR-45(OH) resins before examining by paper chromatography and electrophoresis in borate buffer. In addition to the glucose and ribose already detected, the presence of small amounts of spots corresponding to maltose, maltotriose and higher maltosaccharides was also indicated. The chromatographic and electrophoretic mobilities are listed below.

	Glucose	Ribose	Maltose	Maltotriose
Chromatography (10:4:3) (R_G)	1.00	1.47	0.69	0.48
Electrophoresis (borate buffer) (M_G)	1.00	0.72	0.28	0.28

Since all these impurities were present in very small amounts, it was not thought that they would interfere with the present experiments. The non-diffusible material had the same hydrolase and transferase properties as the original extract.

3. Hydrolase Activity.

Digests containing substrate (2 mg.), extract 2 (1 mg.) and water (1 ml.) were incubated at 35° and samples withdrawn for examination by paper chromatography using 10:4:3 as solvent.

The results are tabulated below.

Activity	Relative strength (visual estimation)	Activity	Relative strength (visual estimation)
Maltase	very strong	cellobiase	strong
phenyl α-glucosidase	medium	phenyl β-glucosidase	weak
methyl α-glucosidase	weak	methylβ-glucosidase	very weak
		p-nitrophenyl β-glucosidase	medium
invertase	undetectable		
melibiase	weak	lactase	medium
methyl α-xylosidase	weak	xylobiase	weak
dextranase	undetectable	laminarinase	weak
amylase	strong	xylanase	undetectable

4. Transglucosylation with Phenyl α-glucoside as Donor and Various acceptor substrates.

Archibald and Manners (1959) showed that transfer of glucosyl radicals from maltose to various pentoses occurred in the presence of cell-free extracts from Tetrahymena pyriformis. In the present experiments phenyl α-glucoside has been used in place of maltose as donor substrate. Phenyl α-glucoside produces fewer glucose-containing oligosaccharides and it is thus easier to detect new oligosaccharides produced by transfer of glucosyl radicals to various acceptors.

Digests containing phenyl α-glucoside (10 mg.), substrate (10 mg.) and extract 2 (2 mg.) in water (1 ml.) were incubated at 36°

for 2-3 days. The digest was applied to Whatman 3MM paper and developed in 10:4:3. Guide strips were sprayed with silver nitrate reagent and the zones which contained the probable transfer products were eluted with water. The concentrated eluates were examined by paper chromatography (to check that only one component was present in each). Hydrolysis of the component before and after reduction followed by paper chromatography of the neutralised hydrolysates indicated the nature of the oligosaccharide..

Acceptors of glucosyl radicals from phenyl α-glucoside:

D-galactose, D-mannose.

D-xylose, D-ribose, D-lyxose, D-arabinose, L-arabinose.

mannitol, sorbitol, xylitol, arabinitol, erythritol, glycerol, ethylene glycol.

Non-acceptors:

D-fructose, L-fucose, L-rhamnose, D-glucuronic acid, sucrose, methyl α-D-glucoside.

To test for synthesis from monosaccharides.

Digests containing xylose, glucose and glucose+xylose were incubated with extract 2 under "transfer" conditions. Synthesis of oligosaccharides did not occur.

5. Maltose as donor and acceptor.

The oligosaccharides produced by incubating an 18% solution of maltose with extract 1 for 10 days were characterised by Archibald and Manners (1959) as isomaltose, maltotriose, panose, 6^3-α-glucosyl-maltotriose and maltotraose; the reaction had not proceeded to equilibrium. The object of the present experiments was to find the oligosaccharides present at equilibrium and thus to obtain more information on the transfer specificity of the enzyme system. The reaction proceeded faster (a) using the more active extract 2 and (b) if the concentration of maltose was reduced.

A solution containing maltose (50 mg.) and extract 1 (5 mg.) in water (0.5 ml.) was incubated at 36°. Samples were withdrawn at intervals for paper chromatography using 10:4:3 as solvent and silver nitrate spray reagent. Authentic samples were available as standards and the R_G-values agreed closely with those given by Archibald and Manners (1959). After incubation for only 5 mins. transfer products were detected and up to 8 hr. isomaltose, maltotriose and panose were produced in approximately equal amounts. From 8 hr. to 2 days the amount of maltotriose decreased gradually, while the amount of panose and isomaltose increased. From 2 to 5 days the panose decreased and the isomaltose concentration continued to build up. After 5 days substantial quantities of isomaltotriose were produced. Between 15 to 17 days the composition of the mixture did not change. To check that equilibrium was established, extract 2 (2 mg.) was added and incubation continued for a further 3 days. The composition of the

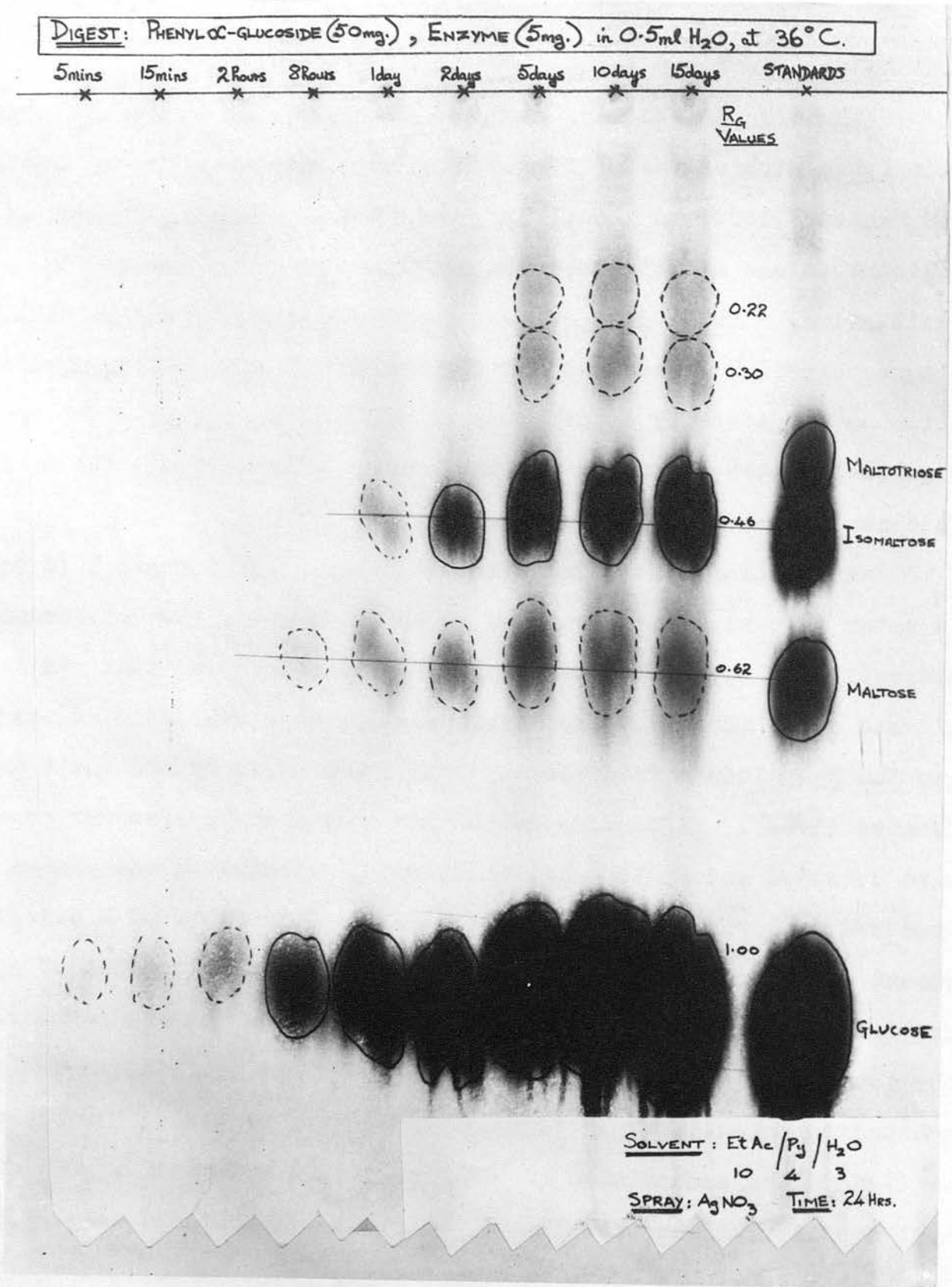

DIGEST: PHENYL α-GLUCOSIDE (50mg.), ENZYME (5mg.) in 0·5ml H_2O, at 36°C.
5mins
15mins
2Hours
8Hours
1day
2days
5days
10days
15days
STANDARDS
R_G VALUES
0·22
0·30
MALTOTRIOSE
0·46
ISOMALTOSE
0·62
MALTOSE
1·00
GLUCOSE
SOLVENT: EtAc / Py / H_2O
10 4 3
SPRAY: $AgNO_3$ TIME: 24 HRS.

FIGURE 18

mixture did not change and glucose (++++), isomaltose (++) and isomaltotriose (+) were the main components. Traces of maltose, maltotriose and panose were barely detectable.

An analogous study was carried out using panose as donor and acceptor. Initially, panose was hydrolysed to maltose and glucose and an oligosaccharide of R_G-value 0.12 was produced (probably 6^3-α-glucosylpanose). As the concentration of glucose increased, isomaltose and isomaltotriose were successively formed and the equilibrium mixture was the same as for the maltose digest.

Starting from isomaltose or isomaltotriose the same equilibrium mixture was obtained, maltose being produced in trace amounts only.

Transfer of glucosyl residues, using maltose as donor and acceptor, occurred even when the concentration of maltose was as low as 0.5%. At 0.1% maltose concentration transfer products could only be detected with difficulty within the first 15 min. of the reaction. As the concentration of maltose was lowered the only transfer product detected initially was panose; this was later replaced by isomaltose, thus emphasising that the primary hydroxyl group is the most favourable acceptor site.

6. Phenyl α-glucoside as donor and acceptor.

A digest containing phenyl α-glucoside (50 mg.), extract 2 (5 mg.) in water (0.5 ml.) was incubated at 36°. The results are shown in Figure 18. By reducing the concentration of phenyl α-glucoside by half, the amount of maltose produced was barely detectable even after

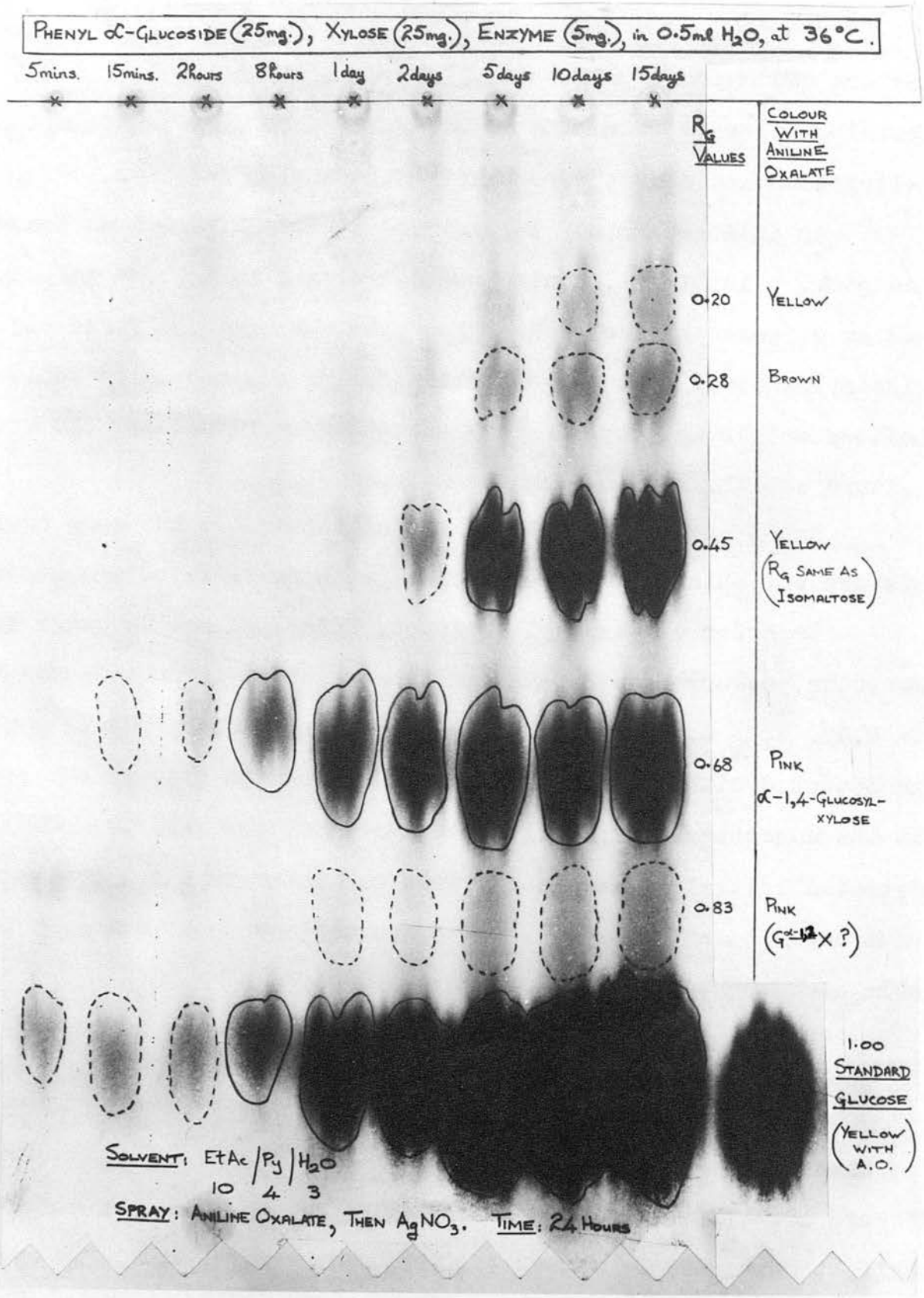

Phenyl α-Glucoside (25mg.), Xylose (25mg.), Enzyme (5mg.), in 0.5ml H_2O, at 36°C.
5mins.
15mins.
2hours
8hours
1day
2days
5days
10days
15days
R_G Values
Colour With Aniline Oxalate
0.20
Yellow
0.28
Brown
0.45
Yellow
(R_G same as Isomaltose)
0.68
Pink
α-1,4-Glucosyl-xylose
0.83
Pink
(G α-1,2 X ?)
1.00
Standard Glucose
(Yellow with A.O.)
Solvent: EtAc / Py / H_2O
10 4 3
Spray: Aniline Oxalate, Then $AgNO_3$. Time: 24 Hours

FIGURE 19

incubation for 10 days although small amounts of isomaltose were still produced. No products resulting from transfer of glucosyl radicals to phenyl α-glucoside were detected. Had any such transfer products been produced they would be expected to have R_G-values greater than that of glucose but less than that of phenyl α-glucoside. Phenyl α-glucoside itself could be detected either by prolonged treatment with silver nitrate reagent or with periodate-permanganate reagent.

7. Preparation and Properties of Glucosylpentoses.

For the preparation of glucosylpentoses, phenyl α-glucoside was chosen as donor of glucosyl radicals, since the only sugar formed in substantial amounts on incubation of phenyl α-glucoside is isomaltose, whereas with maltose significant amounts of several glucose-containing oligosaccharides are produced.

(a) Preparation and Properties of Glucosylxylose.

A preliminary digest containing phenyl α-glucoside (25 mg.), xylose (25 mg.) and extract 2 (5 mg.) in water (0.5 ml.) was incubated at 36° and samples withdrawn at intervals for examination by paper chromatography (see Figure 19). The results indicated that it was possible to stop the digest at a time when there was a substantial amount of the glucose-xylose disaccharide but only traces of glucose containing oligosaccharides. This situation was reached after 1 day using extract 2 but with extract 1, 8 days were required.

A digest containing phenyl α-glucoside (4.5 g.), xylose (5 g.) and extract 1 (1 g.) in water (100 ml.) was incubated at 36° for 8 days. The solution was heated at 100° for 20 min., cooled and centrifuged. Chromatographic examination of the digest before and after this treatment indicated no change in the constituent sugars. The supernatant solution was evaporated to small volume and applied to a charcoal column. The column was prepared as described by Andrews, Hough and Powell (1956) and contained 50 g. of B.D.H. charcoal packed on a paper pulp base in an 8 cm. diameter No. 3 sinter glass filter. The column was eluted with water and 200 ml. fractions collected. The fractions were concentrated to small volume and analysed by paper chromatography using 10:4:3 as solvent. Fraction 1 contained glucose, xylose and a trace of glucosylxylose. Fraction 3 contained traces of the hexoses and some glucosylxylose and fraction 4 contained glucosylxylose and a trace of xylose. Fractions 5 and 6 contained glucosylxylose and a trace of a sugar which gave a pink colour with aniline oxalate and had an R_G-value of 0.83 in 10:4:3. The remainder of the glucosylxylose was eluted from the column with 5% ethanol (700 ml.). Fractions 3 to 6 and the final 5% ethanol fraction were combined and concentrated to a volume of about 0.5 ml. In addition to the sugars already mentioned, traces of a spot corresponding in R_G-value and colour with aniline oxalate to isomaltose were detected. The solution was applied to sheets of Whatman 3MM paper and developed for 24 hr. in 10:4:3. Guide strips were sprayed with aniline

oxalate and the main disaccharide zone eluted with water. The eluate was evaporated to a thin syrup and ethanol added and after about 5 min. a white solid was formed. The material was washed with ether and dried under vacuum at room temperature (yield 231 mg.).

The zone containing the sugar of R_G-value 0.83 was eluted. The sugar ran as a single spot on paper chromatography (10:4:3 as solvent) and gave a pink colour with aniline oxalate fluorescing a bright orange colour under ultraviolet light. On electrophoresis in borate buffer it gave three spots M_G-values 0.64, 0.48 and 0.28 (all gave pink with aniline oxalate; the latter major spot fluoresced under U.V. light). The amount of these trace sugars was so small that no more material was available for further tests.

Examination of Glucosylxylose.

The sugar ran as a single spot on paper chromatography using 10:4:3, 18:3:1:4 and 4:1:5 as solvents. The specific rotation was $[\alpha]_D^{16}$ + 101° after 10 min. decreasing to +97° after 100 min. (c 0.89, water).

On total acid hydrolysis glucose and xylose were formed in equal amounts (visual estimation) and on reduction followed by acid hydrolysis glucose and xylitol were produced, indicating that the xylose residue is in the reducing position.

Electrophoresis.

The disaccharide had an M_G-value of 0.26 in borate buffer (20 volts./cm., 40 min.) suggesting that the linkage is either

(1→2) or (1→4). In analogous experiments with maltose, laminaribiose, isomaltose and 3-O-β-D-glucopyranosyl-D-xylose the M_G-values were 0.28, 0.56, 0.55 and 0.64 respectively (see Figure 26[a] facing p.173).

The reduced disaccharide had an M_S-value of 0.30 in molybdate buffer (cf. cellobiitol and maltitol, 033; laminaribiitol 0.00). This also suggests a (1→2) or (1→4) linkage (see Figure 26[a] facing p. 173).

Selective Spray Reagents.

The disaccharide gave pink colours with aniline oxalate, aniline hydrogen phthalate and p-anisidine hydrochloride spray reagents.

With triphenyltetrazolium chloride a rich pink colour was formed after 40 sec. (Sophorose did not produce any colour even after 10 min.). This provides tentative evidence that the linkage is not (1→2).

The disaccharide and the derived alcohol both gave a blue colour with p-rosaniline spray reagent. An authentic sample of 3-O-β-D-glucopyranosyl-D-xylopyranoside gave a yellow colour with this reagent and a (1→2)-linked glucosylxylose would also be expected to give a yellow colour (see Figure 25 facing page 175).

Estimation of Periodate Consumption.

Glucosylxylose (10.4 mg.) was dissolved in 0.015M-sodium metaperiodate (25 ml.) and samples (1 ml.) withdrawn at intervals for estimation of periodate consumption by the method of Aspinall and Ferrier (1957) as described in the experimental methods section.

The reaction was carried out at room temperature.

Results:	Time (hr.)	Periodate consumption*	Time (hr.)	Periodate consumption*
	0.75	1.65	18	3.75
	1.5	1.89	20.5	3.86
	7.5	3.19	24.5	3.86
	8.5	3.22	27	3.86 constant.

* Periodate consumption is expressed in mol. prop./glucosyl-xylose molecule.

The value is in close agreement with the theoretical value of 4.00 mol.prop. expected from a (1→4) or (1→5) linked glucosyl-xylose.

Action of an Almond Emulsin Preparation.

An almond emulsin preparation, prepared by Cunningham (1961) was tested for α- and β-glucosidase activities. The preparation (1 mg.) was incubated with substrate (2 mg.) and water (0.1 ml.) at 36° and samples tested after 1 day by paper chromatography using 10:4:3 as solvent and silver nitrate spray reagent.

Substrate	Hydrolysis	Substrate	Hydrolysis
cellobiose	+	maltose	-
salicin	+	isomaltose	-
3-O-β-D-glucopyranosyl-D-xylopyranose	+	α-glucosylxylose	-
β-glucosylxylose*	+		

* The β-linked glucosylxylose was a sample obtained by transfer of glucosyl radicals from salicin to xylose by a barley β-glucosidase preparation. The β-linked glucosylxylose was examined in the present investigation and the results are compared with the β-linked glucosylxylose in Table 5 (page 173).

The lack of hydrolysis by almond emulsin indicates that the glucosylxylose contains an α-glucosidic linkage.

Methylation.

Glucosylxylose (5 mg.) was methylated by the method of Kuhn, Trischmann and Löw (1955) and the product divided into two portions. The first portion was hydrolysed with sulphuric acid, neutralised with barium carbonate and examined by paper chromatography using 200:17:1 as solvent and aniline oxalate spray reagent. Two major

components with R_F-values 0.49 and 0.78 were detected. Authentic samples of 2,3-di-O-methyl-D-xylose, 2,4-di-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose had R_F-values of 0.49, 0.41 and 0.78 respectively.

The second portion of the methylated product was treated with a 3% solution of methanolic hydrogen chloride as described in the experimental section and product examined by gas-liquid chromatography (performed by Dr. G.O. Aspinall). Peaks corresponding to the mixed methyl glycosides of 2,3-di-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose were produced.

These preliminary methylation experiments suggest that the disaccharide contains a (1→4) or (1→5)-glucosidic linkage. The methylation was carried out on a larger scale to obtain a full characterisation of the methylated sugars. The disaccharide (60 mg.) dissolved in dimethyl formamide (10 ml.) was methylated with methyl iodide (10 ml.) and silver oxide (1 g.) as described in the experimental methods section. The methylation was repeated and the product recovered in 60% yield. The methylated disaccharide was hydrolysed, neutralised and applied to a small cellulose column (Whatman standard grade cellulose). The column was eluted with methyl ethyl ketone/water (10:1, by vol.) at a rate of 6 ml./hr. and fractions (2 ml.) collected. Fractions were analysed by paper chromatography using 200:17:1 as solvent and aniline oxalate spray reagent. Fractions 5 and 6 contained a single component corresponding in chromatographic mobility with 2,3,4,6-tetra-O-methyl-D-glucose.

Fractions 7 and 8 contained 2,3-di-O-methyl-D-xylose and traces of tetramethylglucose; in addition a trace spot running slightly faster than 2,3-dimethylxylose was detected. Fractions 9-12 contained the dimethylxylose. Subsequent fractions contained traces of undermethylated products.

Fractions 5 and 6 were evaporated to a syrup (22 mg.) which crystallised after a period of 2 days. The product was recrystallised three times from petroleum ether (b.p.60-80°) to yield colourless crystals (16 mg.) m.p. and mixed m.p. 83-84°, $[\alpha]_{17}^{D}$ + 85° (c 0.5, H_2O). The crystals gave an X-ray powder photograph (taken by Mr. K. Fraser) identical with that of an authentic sample of 2,3,4,6-tetra-O-methyl-D-glucose.

Fractions 7-10 were purified by preparative chromatography and combined with fractions 11 and 12 to yield a pale yellow syrup (15 mg.) which did not crystallise. The syrup was seeded with an authentic sample of 2,3-di-O-methyl-D-xylose (β-form). The crystalline mass, which formed overnight, was washed free of non-crystalline, yellow material with a small volume of cold ethyl acetate. The clear crystals (11 mg.) had m.p. and mixed m.p. 78-81° and gave an X-ray powder photograph (taken by Mr. K. Fraser) identical with that of an authentic sample of 2,3-di-O-methyl-D-xylose (β-form) and different from 2,4-di-O-methyl-D-xylose.

Selective Periodate Degradation of Glucosylxylose.

The periodate and methylation studies are consistent with the disaccharide containing a (1→4)-linkage. However, a glucosylxylose with a (1→5)-linkage would also yield 2,3,4,6-tetra-O-methyl-D-glucose and 2,3-di-O-methyl-D-xylose on methylation analysis and would consume 4.0 mol.prop. of periodate. Electrophoresis in molybdate buffer gave tentative evidence that a (1→5)-linkage was absent and the following scheme provides confirmatory evidence for this (see Figure 24,facing p.174).

The glucosylxylose (20 mg.) was dissolved in water (20 ml.) and potassium borohydride (10 mg.) added. The mixture was allowed to stand for 12 hr. and then the excess borohydride destroyed with Amberlite IR-120(H) resin. Borate was removed by successive evaporations with methanol and the solution adjusted to pH 4. The product was oxidised with sodium metaperiodate (40 mg.) in a total volume of 250 ml. at room temperature for 24 hr. The solution was deionised with Amberlite IR-120(H) and IR-45(OH) resins before concentrating to a volume of about 20 ml. Borohydride reduction was carried out as previously described and the product hydrolysed with sulphuric acid. Glucose and glycerol were the only major spots detected on paper chromatography. In an analogous experiment with isomaltose, glucose and ethylene glycol were formed; a (1→5)-linked glucosylxylose would also yield ethylene glycol (see Figure 24, facing p.174).

Preparation and Properties of Glucosylribose.

A digest containing phenyl α-glucoside (4.5 g.), ribose (5 g.) and extract 1 (1 g.) in water (100 ml.) was incubated at 36° for 8 days. The digest was tested and treated exactly as described for the preparation of glucosylxylose (p.138), except that the column contained Ultrasorb S.C. 120/124 charcoal in place of B.D.H. charcoal.

The column was eluted with water (6.25 l.) followed by 2% ethanol (4.25 l.); the eluates contained only glucose and ribose. The third fraction (2% ethanol, 1 l.) did not contain sugars. The glucose-ribose disaccharide was eluted from the column with 14% ethanol (1 l.). Subsequent 14% ethanol fractions did not contain sugars. The disaccharide fraction was concentrated and dried to an amorphous powder (200 mg.). Paper chromatography indicated the presence of one main component with an R_G-value of 0.76 (10:4:3 as solvent) and giving a pink colour with aniline oxalate. Trace spots corresponding to isomaltose and a pentose-containing disaccharide of R_G-value 0.85 (10:4:3) were also detected. The trace spots were separated from the main disaccharide by thick paper chromatography. The trace spot (R_G 0.85) gave a yellow colour with periodate-p-rosaniline reagent before reduction and a blue colour after reduction; it did not react with the triphenyl tetrazolium chloride reagent and gave a peculiar orange fluorescence with aniline oxalate reagent under ultraviolet light. It is therefore thought to consist mainly of a (1→2)-linked glucosylribose.

Examination of Main Glucose-Ribose Disaccharide.

The disaccharide ran as a single spot in 10:4:3 and 18:3:1:4 as solvents, the R_G-values being 0.76 and 0.58 respectively. The specific rotation of the dried amorphous powder was $[\alpha]_D^{16}$ + 93° (c 0.95, water).

On total acid hydrolysis glucose and ribose were formed in approximately equal amounts (visual estimation) and on reduction followed by acid hydrolysis glucose and ribitol were produced, indicating that the ribose residue is in the reducing position.

Electrophoresis.

The glucosylribose had an M_G-value of 0.28 in borate buffer (cf. maltose, 0.28). The reduced disaccharide had an M_S-value of 0.01, eliminating the possibility of a (1→5)-linkage.

Selective Spray Reagents.

The disaccharide gave pink colorations with aniline oxalate, aniline hydrogen phthalate and p-anisidine hydrochloride spray reagents.

With triphenyl tetrazolium chloride a rich pink colour was formed almost immediately, suggesting that the linkage is not (1→2).

The disaccharide gave a blue colour with p-rosaniline spray reagent. (A (1→2) or a (1→3)-linked glucosylpentose would give a yellow colour, due to the formation of malondialdehyde structures, as shown in Figure 25, facing p. 175.

Estimation of Periodate Consumption.

Glucosylribose (10.6 mg.) was dissolved in 0.015M-sodium metaperiodate (25 ml.) and samples (1 ml.) withdrawn at intervals for estimation of periodate consumption by the method of Aspinall and Ferrier (1957). The reaction was carried out at room temperature.

Results:

Time (hr.)	Periodate consumption*	Time (hr.)	Periodate consumption*
0.5	2.06	10	3.61
1.5	2.75	21	3.72
3	2.93	31	3.72
6	3.48		

* Periodate consumption is expressed in mol.prop./glucosylribose molecule.

The above results confirm the absence of (1→2) or (1→3)-linkages. The disaccharide is therefore 4-O-α-D-glucopyranosyl-D-ribose.

Preparation and Properties of Glucosylarabinoses.

A digest containing D-arabinose (5 g.), phenyl α-glucoside (4.5 g.) extract I (1 g.) in water (100 ml.) was incubated at 36° for 8 days. The solution was heated at 100° for 20 min. and the denatured protein centrifuged down. The solution was examined before and after this treatment and no change was observed in the products. The supernatant solution was concentrated and applied to a charcoal column as described for the preparation of glucosyl-xylose (p.138). The column was eluted with water and fractions (200 ml.) collected. Fractions 1-3 contained glucose and arabinose. Fractions 4-6 contained glucosylarabinose(s), a trace of isomaltose and a trace of arabinose. 5% ethanol (600 ml.) was used to complete the elution of the disaccharides.

The mixture of arabinose-containing disaccharides was applied to thick paper and developed in 10:4:3. The zones containing the traces of isomaltose and arabinose were removed and the remaining disaccharides separated into two fractions. The fraction with a low chromatographic mobility is referred to as fraction A and the fast moving fraction as fraction B.

Fraction A (obtained as an amorphous powder, 160 mg.) separated into three spots when chromatographed with 10:4:3 as solvent. The R_G-values and colours with aniline oxalate and p-rosaniline spray reagents are listed below.

Component	R_G-value (10:4:3)	Aniline oxalate Daylight	Aniline oxalate U.V.	p-rosaniline
1	0.61	pink	maroon	blue
2	0.70	pink	maroon	yellow
3	0.77	pink	orange (fluorescent)	yellow

An authentic sample of 3-O-α-D-glucopyranosyl-D-arabinopyranose had an R_G-value of 0.70 and gave a yellow colour with p-rosaniline.

Methylation of Fraction A.

Fraction A (5 mg.) was methylated by the method of Kuhn, Trischmann and Löw (1955) as described in the experimental methods section. The product was methanolysed with 3% methanolic hydrogen chloride and examined by gas chromatography. Peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucose; 2,3, -di-O-methyl-D-arabinose and 3,4-di-O-methyl-D-arabinose were produced.

On electrophoresis in borate buffer, fraction A produced two spots with M_G values 0.31 and 0.45, and in molybdate buffer the borohydride reduced mixture produced an elongated spot covering M_S-values from 0.00 to 0.25.

Fraction A was hydrolysed by acid to glucose and arabinose in approximately equimolecular proportions (visual estimation) and on reduction followed by hydrolysis glucose and arabinitol were formed. The mixture was not hydrolysed by almond emulsin enzyme preparation.

One possible interpretation of the above results is that fraction A contains three glucosylarabinoses, the linkage in each being as follows: component 1, α-(1→4); component 2, α-(1→3); component 3, α-(1→2).

Fraction B (obtained as an amorphous powder, 30 mg.) separated into two components, R_G-values 0.77 and 0.89 in 10:4:3. Since the

former component appears to be common to fraction A and fraction B, it is hereinafter referred to as component 3 and the latter is referred to as component 4. Component 3 gave the same colour reactions as component 3 from fraction A. The peculiar orange fluorescence which component 3 produced under ultraviolet light when sprayed with aniline oxalate was at first thought to be due to the presence of an arabinofuranose reducing end-group, but an authentic sample of 5-O-α-D-glucopyranosyl-D-arabinose, in which the arabinose unit must be in the furanose form, gave a maroon colour under ultra-violet light. The orange fluorescence is probably due to the presence of a (1→2)-linkage. Confirmation of this was obtained by spraying a chromatogram of fraction B with triphenyl tetrazolium chloride. Component 3 gave no colour, whereas component 4 produced a rich pink colour almost immediately. Component 4 had an R_G value identical to that of an authentic sample of 5-O-α-D-glucopyranosyl-D-arabinose and gave a blue colour with p-rosaniline spray.

Methylation of Fraction B.

Fraction B (5 mg.) was methylated and methanolysed as previously described. The product was examined by gas chromatography and peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucose; 2,3-di-O-methyl-D-arabinose and 3,4-di-O-methyl-D-arabinose were detected.

Fraction B separated into two components on electrophoresis in borate buffer. The major component had an M_G-value of 0.45 and the

minor component an M_G-value of 0.31. Reduction of fraction B followed by electrophoresis in molybdate buffer produced two spots; the major spot had an M_S-value of 0.43 (cf. 5-O-α-D-glucopyranosyl-D-arabinitol, 0.41) and the minor component an M_S-value of 0.01. Fraction B was hydrolysed by acid to glucose and arabinose in approximately equimolecular proportions (visual estimation) and on reduction followed by acid hydrolysis, glucose and arabinitol were formed. Fraction B was not hydrolysed by an almond emulsin enzyme preparation.

These results on fraction B suggest that component 3 is 2-O-α-D-glucopyranosyl-D-arabinopyranose and that component 4 is 5-O-α-D-glucopyranosyl-D-arabinose.

Separation of the four disaccharides, which differed in chromatographic mobility only by very small amounts in all the available solvents, proved difficult. However, by repeated preparative paper chromatography a few milligrams of each component was obtained chromatographically pure. The disaccharides gave the colour reactions previously described and on reduction followed by treatment with periodate-p-rosaniline the following colours were produced: component 1, blue; component 2, yellow; component 3, blue; component 4, blue.

On electrophoresis in molybdate buffer the mobilities of the reduced compounds were as follows:- component 1, 0.22; component 2, 0.00; component 3, 0.00; component 4, 0.45. (The theoretical interpretation of these values is indicated on Figure 26[b], facing p. 178).

The following tentative structures are therefore suggested.

Component 1 4-O-α-D-glucopyranosyl-D-arabinopyranose.

2 3-O-α-D-glucopyranosyl-D-arabinopyranose.

3 2-O-α-D-glucopyranosyl-D-arabinopyranose.

4 5-O-α-D-glucopyranosyl-D-arabinofuranose.

Preparation and Properties of Glucosyllyxoses.

A digest containing phenyl α-glucoside (2.25 g.), D-lyxose (2.5 g.) and extract 2 (0.5 g.) in water (50 ml.) was incubated at 36° for 4 days. The mixture was heated at 100° for 20 min. to stop the enzyme action. Chromatographic examination before and after this heat treatment indicated no change in the products. The mixture was concentrated to half its original volume, filtered through glass wool to give a clear, pale yellow liquid, which was concentrated to a volume of ca 10 ml. The solution was applied to a column consisting of a mixture of charcoal (30 g.) and Celite (30 g.). The charcoal used was Ultrasorb S.C. 120/124 (British Carbo-Norit Union Ltd., Grays, Essex). Fractions were analysed by paper chromatography using 10:4:3 as solvent and aniline oxalate spray reagent.

Fr.	Eluent	Vol. (ml.)	R_G-value (10:4:3)	Colour with A.O.	Probable contents
A	Water	1100	1.00	yellow	glucose (+)
			1.61	pink	lyxose (++++)
B	Water	1650	1.00	yellow	glucose (+)
			1.61	pink	lyxose (±)
C	Water	1100	1.00	yellow	glucose (+)
D	Water	2200	1.00	yellow	glucose (±)
E	Water	2200	1.70	pink	ribose (±)
F	2% ethanol	1650	1.70	pink	ribose (±)
G	4% ethanol	1100	0.70	pink	disaccharide (a) (+++)
H	4% ethanol	550	0.70	pink	disaccharide (a) (++)
			0.96	pink	disaccharide (b) (+)
I	4% ethanol	550	0.70	pink	disaccharide (a) (±)
			0.96	pink	disaccharide (b) (+)
			0.43	yellow	isomaltose (±)
J	4% ethanol	3300	0.70	pink	disaccharide (a) (±)
			0.96	pink	disaccharide (b) (+)
			0.87	pink	disaccharide (c) (+)
			0.43	yellow	isomaltose (±)
K	6% ethanol	1100	0.43	yellow	isomaltose (±)
L	30% ethanol	2000	Traces of higher oligosaccharides.		

Fractions E and F contained a sugar which had the same chromatographic and electrophoretic mobilities as D-ribose. The sugar, which gave a yellow colour with aniline oxalate in fractions I, J and K, after purification by thick paper chromatography had the same chromatographic and electrophoretic mobility as isomaltose. These sugars were not examined further.

The component sugars of fractions H, I and J were separated by successive developments on thick paper using 10:4:3 as solvent.

The disaccharide of R_G-value 0.70 was combined with fraction G and the following yields obtained: disaccharide (a) 147 mg., disaccharide (b) 60 mg., disaccharide (c) 29 mg. (weighed as dry amorphous powders).

Examination of disaccharides (a), (b) and (c).

Each disaccharide was chromatographically and electrophoretically pure. Acid hydrolysis yielded glucose and lyxose in approximately equimolecular proportions and reduction followed by acid hydrolysis yielded glucose and lyxitol (arabinitol) in each case. The disaccharides were unattacked by a β-glucosidase preparation from almond emulsin and had high positive specific rotations (see Table 4) suggesting the presence of α-glucosidic linkages.

The chromatographic and electrophoretic mobilities, the colours with selective spray reagents and the specific rotations are listed in Table 4. The probable linkage of each disaccharide is also indicated.

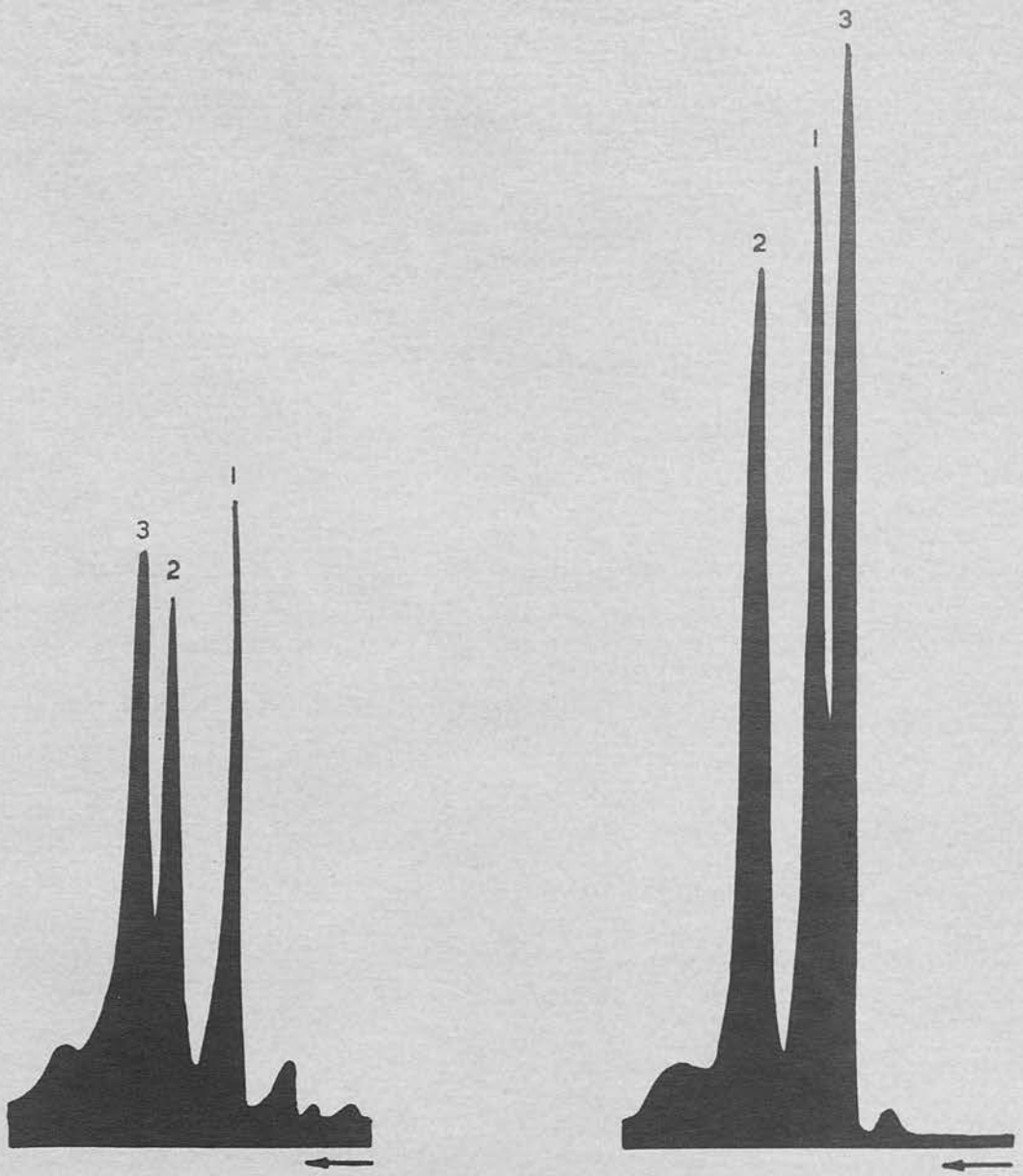
TABLE 4.

Properties of Some Glucosyllyxoses.

Disaccharide	R_G (10:4:3)	R_G (18:3:1:4)	M_G (borate buffer)	M_S (reduced disaccharide) (molybdate buffer)
(a)	0.70	0.44	0.45	0.04
(b)	0.97	0.68	0.25	0.20
(c)	0.89	0.59	0.45	0.06

Disaccharide	Colour with p-rosaniline: Before reduction	Colour with p-rosaniline: After reduction	Reaction with triphenyl tetrazolium chloride	Colour with aniline oxalate (U.V.light)	Probable linkage
(a)	blue	blue	+	maroon	1→4
(b)	yellow	blue	-	orange (fluorescent)	1→2
(c)	yellow	yellow	+	maroon	1→3

GAS CHROMATOGRAMS OF METHYL GLYCOSIDES FROM METHYLATED GLUCOSYLLYXOSE



(a) Neopentylglycol adipate
150°

(b) Polyphenyl ether-
[m-bis-(m-phenoxyphenoxy)-benzene]
200°

Relative retention times of methyl glycosides:-

	Column (a)	Column (b)
1. 2,3,4,6-Tetra-O-methyl-D-glucose	1.00	1.00
2. 2,3,4,6-Tetra-O-methyl-D-glucose	1.44	1.30
3. 2,3-Di-O-methyl-D-lyxose	1.70	0.87
Authentic 2,3-di-O-methyl-D-lyxose	1.69	0.88

FIGURE 20

Methylation of glucosyllyxoses.

The disaccharides (5 mg. each) were methylated by the method of Kuhn, Trischmann and Löw (1955) and the products divided into two portions. The first portion was hydrolysed and examined by paper chromatography. In each case tetramethylglucose and one other major spot were produced in approximately equal proportions. The R-tetramethylglucose-values in 200:17:1 as solvent and the colours of the second components with aniline oxalate were disaccharide (a) 0.47 (brown), disaccharide (b) 0.50 (pink), disaccharide (c) 0.56 (pink). An authentic sample of 2,3-di-O-methyl-D-lyxose had an R-tetramethylglucose-value of 0.47 and gave a brown colour with aniline oxalate.

It has been found that all methylated sugars with hydroxyl groups on C-4 give brown colours with aniline oxalate, while those with methoxyl groups on C-4 give pink colours. This is consistent with disaccharide (a) being 4-O-α-D-glucopyranosyl-D-lyxose and suggests that disaccharides (b) and (c) contain lyxopyranose moieties; i.e. C-4 probably has a methoxyl group. Further indication that disaccharide (a) was 4-O-α-D-glucopyranosyl-D-lyxose was obtained by methanolysis of the methylated disaccharide and examination of the mixed methyl glycosides by gas-liquid chromatography. Peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucose and 2,3-di-O-methyl-D-lyxose were detected (see Figure 20.).

The dimethyllyxoses were separated by thick paper chromatography and reduced with potassium borohydride. After deionisation, the reduced sugars gave no colour with aniline oxalate. The solutions (0.01 ml.each) were treated with 0.3M-sodium metaperiodate (0.01 ml.) for 24 hr. The products were treated with aniline oxalate spot-tests. The products derived from disaccharides (a) and (b) gave a pink colour, while the products from (c) gave no reaction. A (1→3)-linked glucopyranosyllyxofuranoside would yield a partly methylated lyxose with adjacent hydroxyl groups on C-3 and C-4 and would be susceptible to periodate oxidation, with the production of an aldehyde group on C-4. A positive reaction with aniline oxalate would result. The lack of reaction with aniline oxalate therefore provides further evidence that disaccharide (c) is 3-O-α-D-glucopyranosyl-D-lyxopyranoside.

Preparation and Properties of a Glucosylerythritol.

A digest containing erythritol (5 g.), phenyl α-glucoside (4.5 g.) extract 2 (1 g.) and water (100 ml.) was incubated at 37° for 4 days. The solution was heated at 100° for 20 min. concentrated to a volume of about 10 ml. and applied to a charcoal "column" (Andrews, Hough and Powell, 1956). The charcoal used was Ultrasorb S.C. 120/124 (British Carbo-Norit Union Ltd., Grays, Essex). The fractions were analysed by paper chromatography using 10:4:3 as solvent.

Fr.	Eluent	Volume	R_G-values	Probable contents
A	Water	1200 ml.	1.50	Erythritol
B	Water	1200 ml.	1.50 (1.00)	Erythritol + glucose (trace)
C	Water	1200 ml.	(1.50),1.00	Erythritol (trace) + glucose
D	2% Ethanol	1200 ml.	1.00	Glucose
E	2% Ethanol	2500 ml.	1.00	Glucose
F	5% Ethanol	1000 ml.	-	-
G	10% Ethanol	1000 ml.	0.76	Glucosylerythritol
H	14% Ethanol		0.45 (0.76)	Isomaltose + glucosyl-erythritol(trace)

Examination of Fraction G. (yield 150 mg.)

Fraction G ran as a single spot on paper chromatography using 10:4:3, 18:3:1:4 and 4:1:5 as solvents. The R_G-values were 0.76, 0.68 and 0.75 respectively. On total acid hydrolysis glucose and erythritol were formed in approximately equimolecular proportions

(visual estimation) and on reduction followed by hydrolysis the same compounds were formed indicating that the erythritol residue is in the reducing position. The material was unattacked by a β-glucosidase preparation from almond emulsin, suggesting the presence of an α-glucosidic linkage. The following series of reactions was carried out to determine whether the glucose residue was attached to the central or terminal positions of the erythritol residue. The glucosylerythritol (20 mg.) was oxidised with sodium metaperiodate (30 mg.) in a total volume of 100 ml. in the dark, at room temperature, for 8 hr. The solution was deionised with Amberlite IR-120 and IR-45 resins, before addition of potassium borohydride (20 mg.) The solution was left for 12 hr. at room temperature and deionised as described in the experimental section. Hydrolysis of the product yielded glucose and ethylene glycol indicating that the glucose residue is attached to a primary alcohol group in erythritol (see Figure 28, facing p. 181).

Preparation and Properties of a Glucosylmannitol.

The glucosylmannitol was prepared in a similar manner to the glucosylerythritol, from a digest containing phenyl α-glucoside (4.5 g.), mannitol (5 g.), extract 2 (1 g.) in water (100 ml.). Charcoal (Ultrasorb) chromatography yielded a fraction, which on acid hydrolysis gave glucose and mannitol. On chromatography, using 10:4:3 as solvent, a single spot was produced (R_G-0.45) but,

using 18:3:1:4 as solvent, a trace of isomaltose was indicated in addition to the main non-reducing sugar. The glucosylmannitol was therefore purified by thick paper separation using this latter solvent. The product (150 mg.) crystallised readily from aqueous ethanol. The crystals had a specific rotation of +90° after 10 min. decreasing to +86° at equilibrium (c 2.0, water). The product was unattacked by a β-glucosidase preparation from almond emulsin, whereas a sample of glucosylmannitol from a partial hydrolysate of laminarin (obtained from Dr. D.H. Hutson) was readily hydrolysed to glucose and mannitol by this preparation.

The electrophoretic mobility in molybdate buffer (M_S 0.70) was identical with M_S value of glucosylmannitol from laminarin, suggesting the presence of four adjacent hydroxyl groups i.e. the linkage is either (1→1) or (1→2).

Confirmation of this was obtained by estimation of the formaldehyde released on periodate oxidation. Glucosylmannitol (1.2 mg.) was oxidised with 4mM-sodium metaperiodate (5 ml.) in a total volume of 10 ml. at 2°. Samples were withdrawn for estimation of formaldehyde after 12, 18 and 24hr. The formaldehyde release was constant and amounted to 0.94 mol. prop. In an analogous experiment with glucosylmannitol from laminarin the yield was 0.96 mol. prop.

Periodate Degradation of Glucosylmannitol.

Glucosylmannitol (20 mg.) was oxidised with sodium metaperiodate (20 mg.) in a total volume of 100 ml. at room temperature for 24hr. The solution was deionised and concentrated. On examination by paper chromatography using 10:4:3 as solvent and aniline oxalate spray reagent, a pink spot (R_G 0.88) (+) and a yellow streak (+++) were formed. The sugar of R_G-value 0.88 was isolated by preparative chromatography; on acid hydrolysis glucose and arabinose were formed and on reduction followed by molybdate electrophoresis the M_G-value was 0.45. This component is probably 5-O-α-D-glucosyl-D-arabinose. The zone which gave a yellow colour with aniline oxalate was isolated and reduced. The major component had an R_G-value of 0.92 (10:4:3) and gave glucose and glycerol on hydrolysis. Further periodate oxidation followed by reduction gave a major component with an R_G-value of 1.20 and on hydrolysis glucose and ethylene glycol were formed. These results suggest that the sugar is 1-O-α-D-gluco-pyranosyl-D-mannitol (see Figure 28, facing p. 181).

Preliminary Investigation of Transfer to Galactose and Mannose.

Digests containing phenyl α-glucoside (10 mg.), extract 2 (2 mg.) and mannose or galactose (10 mg.) in water (1 ml.) were incubated at 36° for 2 days. (The commercial samples of mannose and galactose were impure and they were therefore purified by thick paper chromatography before use). The digests were heated at 100° for 10 min. and then applied to 3MM sheets and developed using 10:4:3

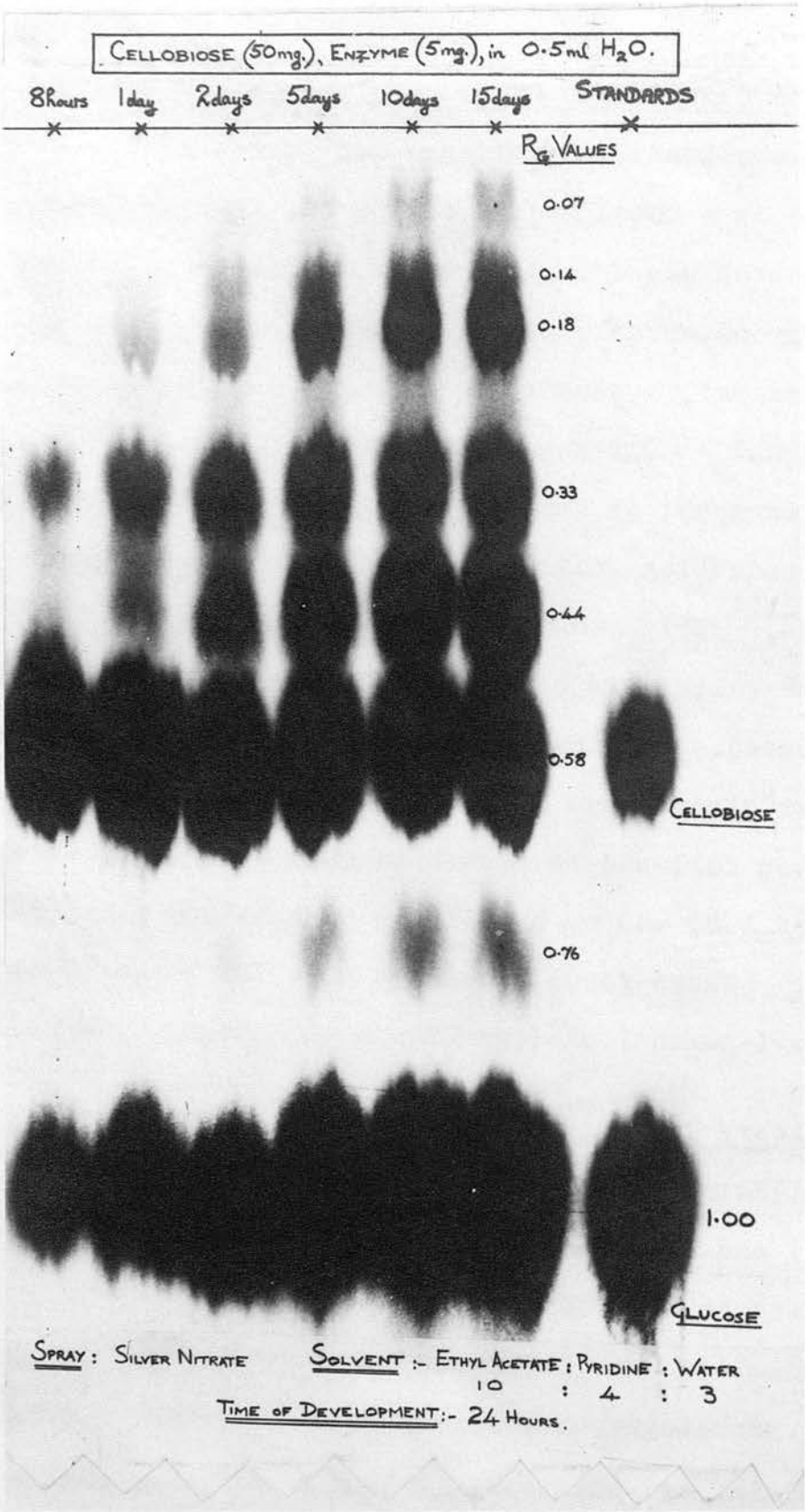

CELLOBIOSE (50mg.), ENZYME (5mg.), in 0.5ml H_2O.
8Hours
1day
2days
5days
10days
15days
STANDARDS
R_G VALUES
0.07
0.14
0.18
0.33
0.44
0.58
CELLOBIOSE
0.76
1.00
GLUCOSE
SPRAY: SILVER NITRATE
SOLVENT :- ETHYL ACETATE : PYRIDINE : WATER
10 : 4 : 3
TIME OF DEVELOPMENT:- 24 HOURS.

FIGURE 21

as solvent. Appropriate disaccharide zones were eluted. The results are tabulated below:-

Digest	R_G of disac.	Colour with p-rosaniline	Products on hydrolysis	Reduction and hydrolysis	M_S-value of reduced Compound.
Mannose	0.52	blue	glucose, mannose	glucose, mannitol	0.70
Galactose	0.33	blue	glucose, galactose	glucose, galactitol	0.70

It therefore seems probable that transfer of glucosyl radicals is occurring to the primary hydroxyl groups (C-6) of both galactose and mannose.

Cellobiose as Donor and Acceptor Substrate.

The results of a preliminary experiment, in which the build up of transfer products was examined at various stages of the reaction, are shown in Figure 21.

A digest containing cellobiose (5 g.), extract 2 (0.5 g,) in water (50 ml.) was incubated at 37° for 15 days. The digest was heated at 100° for 20 min. and denatured protein removed by filtration. About one quarter of the solution was applied to sheets of thick paper and developed in 10:4:3 as solvent. By successive thick paper separations six chromatographically pure fractions were isolated.

All the di- and trisaccharide fractions were readily hydrolysed to glucose by an almond emulsin β-glucosidase preparation, indicating the presence of β-glucosidic linkages. (Maltose, nigerose, isomaltose, maltotriose, isomaltotriose and panose were all unattacked by this preparation.

Examination of fraction 1. (390 mg.)

Fraction 1 was chromatographically and electrophoretically identical to D-glucose. On incubation with a specific D-glucose oxidase reagent (White and Subers, 1961) fraction 1 was completely converted into gluconic acid.

Examination of fraction 2. (10 mg.)

Fraction 2 was chromatographically identical with laminaribiose (R_G 0.76 in 10:4:3). It gave a green colour with diphenylamine-phosphoric acid reagent and a yellow colour with periodate-p-rosaniline reagent before and after reduction. The mobility of the sugar in borate buffer (M_G 0.60) and the mobility of the reduced sugar in molybdate buffer (M_S 0.01) provided further evidence that fraction 2 was laminaribiose.

Examination of fraction 3. (5 mg.)

Fraction 3 had the same chromatographic mobility as sophorose (R_G 0.62 in 10:4:3). On treatment with periodate-p-rosaniline spray reagent the sugar gave a yellow colour, but the reduced compound gave a blue colour, suggesting the presence of a (1→2)-linkage. The electrophoretic mobility of the disaccharide in borate buffer (M_G 0.26) and the mobility of the reduced compound in molybdate buffer (M_S 0.78) provided further evidence for a (1→2)-linkage.

Examination of fraction 4. (150 mg.)

Fraction 4 had the same R_G-value and M_G-value as cellobiose. After reduction it had an M_S-value of 0.30 during molybdate

electrophoresis. The blue colour with diphenylamine-phosphoric acid reagent provided further evidence for the presence of a (1→4)-linkage. Confirmation of this was obtained by methylation followed by methanolysis and examination of the mixed glycosides by gas-liquid chromatography. The presence of 2,3,4,6-tetra-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-glucose was indicated.

Examination of fraction 5. (100 mg.)

Fraction 5 had an R_G-value of 0.44 and an M_G-value of 0.56 in borate buffer. The mobility of the reduced sugar on molybdate electrophoresis (M_S 0.80) suggested the presence of a (1→6)-linkage but did not rule out the possibility of a (1→2)-linkage (Bourne, Hutson and Weigel, 1961). With periodate-p-rosaniline reagent the sugar gave a blue colour. A (1→2)-linked sugar gives a yellow colour with this reagent. Methylation of the sugar on a 5 mg. scale, followed by analysis of the products by the methods described on p.128 , showed the presence of 2,3,4,6-tetra-O-methyl-D-glucose and 2,3,4-tri-O-methyl-D-glucose, confirming the identity of fraction 5 as gentiobiose.

Examination of fraction 6.

Fraction 6 had an R_G-value of 0.33 (10:4:3) and gave a blue colour with diphenylamine-phosphoric acid reagent suggesting the presence of a 4-O-substituted reducing end group. The mobility of the sugar on borate electrophoresis (M_G 0.20) and the mobility of the reduced compound on molybdate electrophoresis (M_S 0.30) provided

further evidence for a 4-O-substituted reducing end-group. Partial acid hydrolysis of fraction 6 yielded spots corresponding to cellobiose, gentiobiose and glucose. Small scale methylation followed by methanolysis and examination of the mixed methyl glycosides by gas-liquid chromatography indicated the presence of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-glucose, suggesting the presence of (1→4) and (1→6)-linkages. Partial acid hydrolysis yielded disaccharide-type spots corresponding to cellobiitol and gentiobiose on paper chromatography using 10:4:3 and 18:3:1:4 as solvents. Fraction 6 is therefore 6^2-O-glucosylcellobiose.

Examination of Higher Oligosaccharides.

A zone of R_G-value 0.18 in 10:4:3 was isolated by preparative chromatography. It was identical to gentiotriose in chromatographic and electrophoretic mobility and in its colour reaction with diphenylamine-phosphoric acid. However, partial acid hydrolysis of the reduced substance yielded several disaccharide-type spots; it is therefore thought that this fraction is a mixture.

The component of R_G-value 0.14 in 10:4:3 gave a blue colour with diphenylamine-phosphoric acid reagent, indicating the presence of a 4-O-substituted reducing end group.

Other experiments on the trans β-glucosylase activity.

1. Starting with gentiobiose, in place of cellobiose, transfer products were synthesised; substantial amounts of cellobiose were formed.

COMPARISON OF SOME HYDROLYTIC ACTIVITIES IN A CELL-FREE EXTRACT FROM TETRAHYMENA.

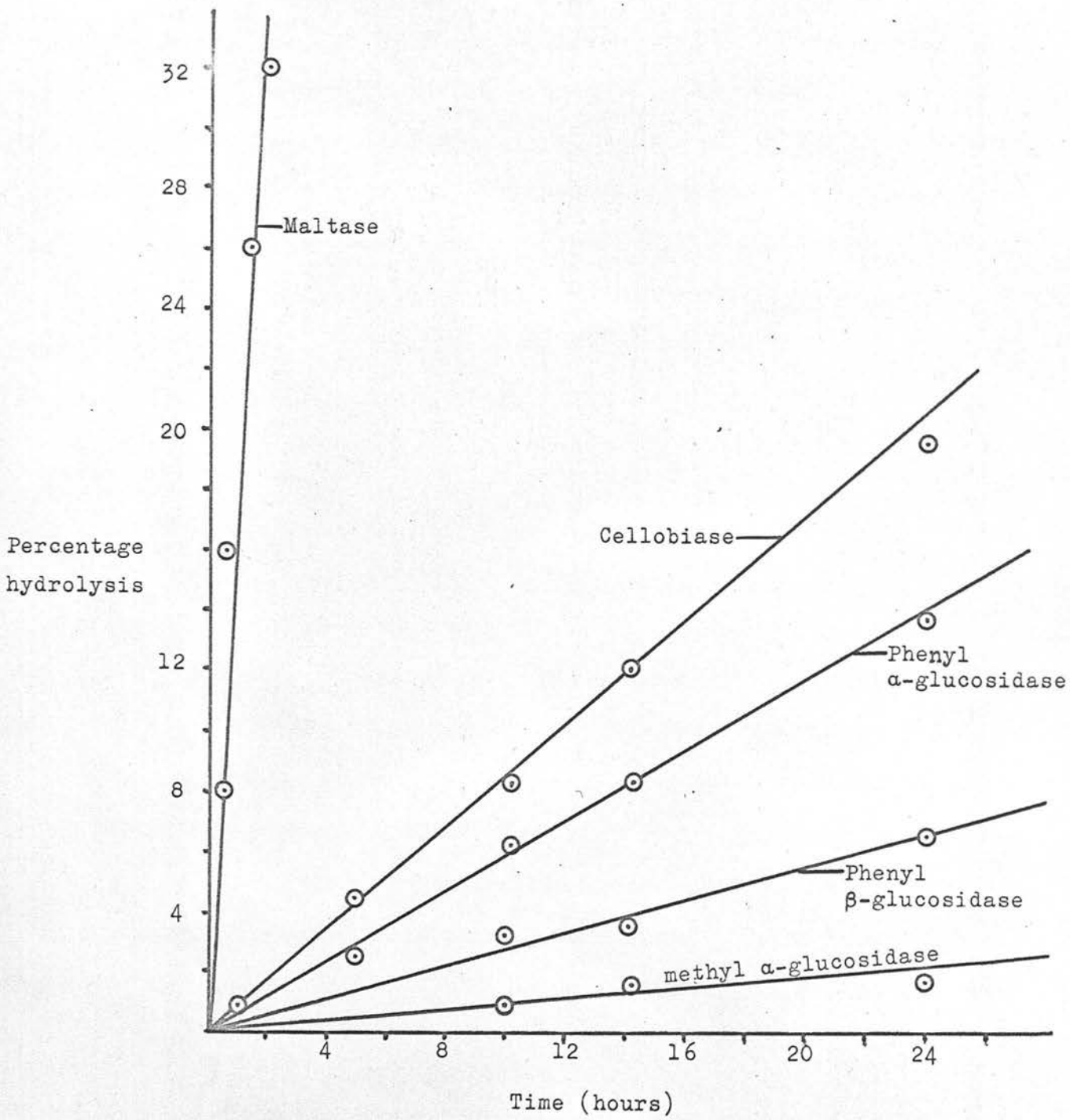


FIGURE 22

2. Using cellobiose as glucosyl donor and D-xylose as acceptor an oligosaccharide with an R_G-value of 0.62 (10:4:3), which gave a pink colour with aniline oxalate, was produced. The same sugar was synthesised on incubation of p-nitrophenyl β-D-glucoside and D-xylose with the enzyme extract.

Quantitative Experiments.

In the following experiments the relative hydrolytic activities were determined and the possibility that the maltase and phenyl-α-glucosidase activities were due to different enzymes investigated.

Comparison of α- and β-Glucosidase Activities.

Digests containing substrate (1 mg./ml.) and enzyme, extract 2, (0.25 mg./ml.) were incubated at 36°. Samples (1 ml.) were withdrawn at intervals for estimation of glucose by the glucose oxidase method (White and Subers, 1961).

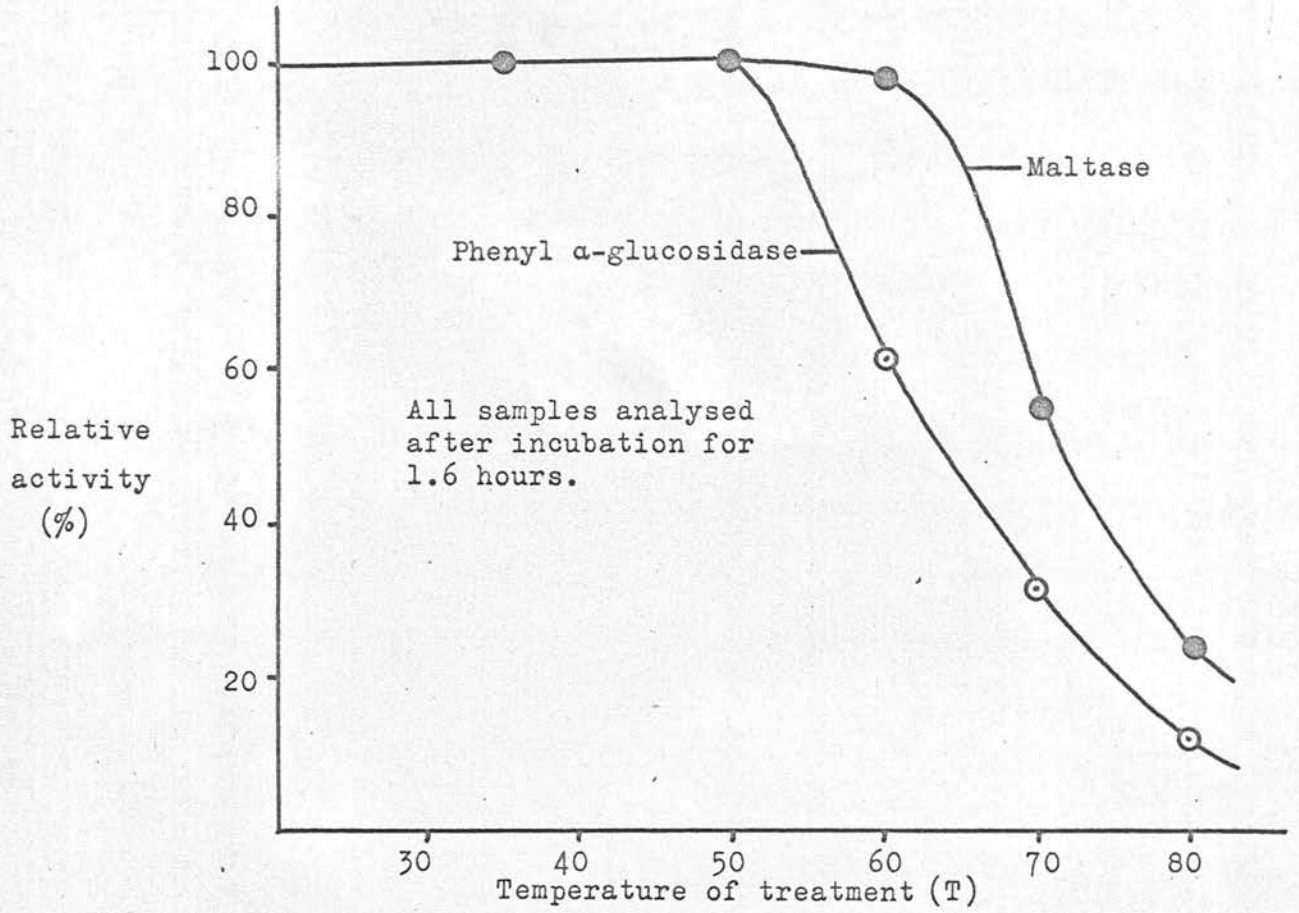
The results are shown graphically in Figure 22. If the maltase activity is taken as 100 units the relative activities are:-

Maltase	100	cellobiase	4.7
phenyl α-glucosidase	3.2	phenyl β-glucosidase	1.5
methyl α-glucosidase	0.5		

Examination of Maltase and Phenyl α-glucosidase Activities.

Digests containing substrate (5 mg.), McIlvaine buffer (2 ml.) and enzyme, extract 2, (1 mg.) in a total volume of 5 ml. were incubated at 36°. Samples (0.5 ml. or 1 ml.) were withdrawn for

HEAT DEACTIVATION



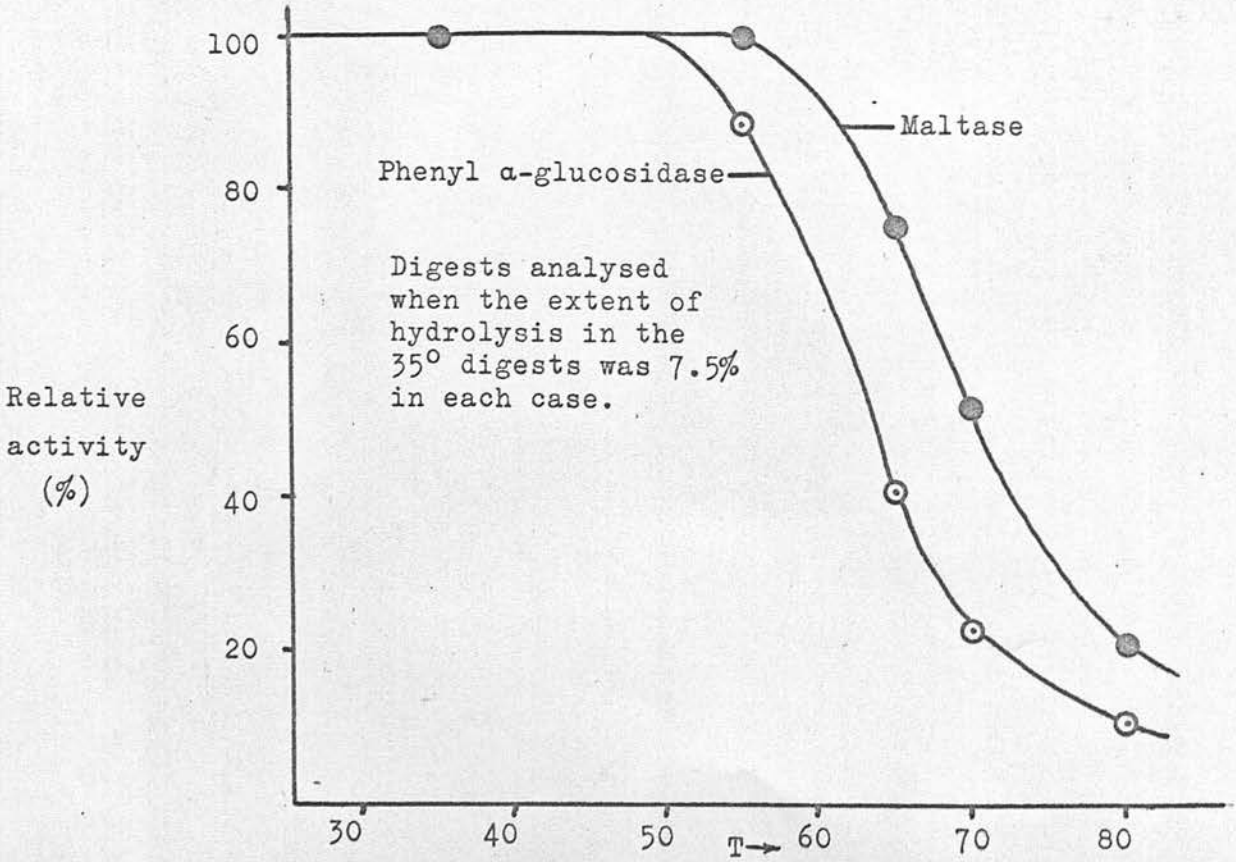


FIGURE 23

estimation of glucose by the glucose oxidase method. The pH-optima were phenyl α-glucosidase 5.0, maltase 4.7.

Heat Deactivation Experiments.

Enzyme, extract 2, (37 mg.) was dissolved in McIlvaine buffer of pH 4.8 (55 ml.). (A small amount of residual protein was removed by centrifugation). The solution was divided into five portions. The first portion was not pretreated; the others were heated for 20 min. at 50°, 60°, 70° and 80° respectively. The solutions were centrifuged to remove denatured protein and digests containing enzyme solution (3 ml.) and substrate (5 mg. in 2 ml. H_2O) prepared. Samples (0.5 ml. of the maltose digest and 2 ml. of the phenyl α-glucoside digest) were withdrawn after 1.6 hr. for estimation of glucose by the glucose oxidase method.

In a second experiment samples were taken from the maltose digest after 1.5 hr. At this stage the percentage of maltose hydrolysed in the non-heat-treated digest was 7.5%. Samples were taken from the phenyl α-glucosidase digest after about 20 hr. when 7.5% of the phenyl α-glucoside was hydrolysed in the "untreated" digest.

The results of both experiments are indicated graphically in Figure 23 and suggest that the maltase and phenyl α-glucosidase activities may be due to different enzymes.

DISCUSSION.

The present experiments have been carried out on cell-free extracts of Tetrahymena pyriformis, prepared by Dr. J.F. Ryley. The extracts contained trace quantities of glucose and maltosaccharides, which may have arisen from enzymic degradation of the storage polysaccharide (glycogen) during the preparation of the extracts; traces of ribose were also detected. The amount of these sugars was so small that it is not thought that they would interfere with the present experiments.

A general survey of the hydrolase activity of two cell-free extracts from Tetrahymena pyriformis indicated that the same activities were present in each, although extract 1 was much weaker than extract 2. In general the α-glucosidase activity was much stronger than the β-glucosidase activity.

Maltose as Donor and Acceptor.

The oligosaccharides produced by incubating an 18% solution of maltose with extract 1 for 10 days were characterised by Archibald and Manners (1959) as isomaltose, maltotriose, panose, 6^3-α-glucosylmaltotriose and maltotetraose; the reaction had not proceeded to equilibrium. In the present investigation it has been shown that transfer, with maltose as donor and acceptor, will occur even when the concentration of maltose is as low as 0.5%. The equilibrium mixture using 10% maltose solution contains glucose, isomaltose and isomaltotriose, but only traces of other sugars, suggesting that

transfer to C-6 of glucose is highly favoured. Archibald (1958) suggested that since the yields of panose and maltotriose were about the same, under the conditions used, the C-4 and C-6 positions of the non-reducing end of maltose are equally favourable acceptor sites. In the present investigation it has been shown that during the initial phases of the reaction panose and maltotriose are present in approximately equal amounts but later in the reaction the maltotriose disappears while the panose continues to increase. Furthermore, using 0.5% - 1% maltose solutions, panose is produced initially, but is later replaced by isomaltose; very little maltotriose is formed. It is therefore thought that C-6 is the most favourable acceptor site.

The isomaltose may be formed by transfer of glucosyl radicals to the C-6 position of glucose or by hydrolysis of the (1→4)-linkage in panose. To distinguish between these two possibilities panose was incubated with extract 2; maltose and glucose were formed in the initial stages of the reaction indicating that the isomaltose is most probably formed by transfer to glucose.

Transfer of Glucosyl Radicals from Phenyl α-Glucoside.

Incubation of a 10% solution of phenyl α-glucoside with the enzyme preparation resulted in the formation of isomaltose and small amounts of maltose (see Figure 18 facing p. 136). By halving the concentration of phenyl α-glucoside, the amount of maltose produced was barely detectable even after incubation for 10 days; small amounts

of isomaltose were, however, produced. Transfer of glucosyl radicals to phenyl α-glucoside itself did not appear to occur.

A general survey of compounds which would act as glucosyl acceptors, using phenyl α-glucoside as donor indicated that D-aldohexoses, D-pentoses and certain sugar alcohols were all suitable acceptors, whereas D-fructose, L-fucose, L-rhamnose, D-glucuronic acid, sucrose and methyl-α-D-glucoside did not appear to satisfy the acceptor specificity requirements of the system.

Since xylose is stereochemically the same as glucose except that it has no $-CH_2OH$ group (the most favourable acceptor site) it was of interest to prepare the glucosylxylose produced and to examine the mode of linkage. It was also thought that examination of the transfer products, formed with other pentoses as acceptors, might yield information on stereochemical influences on the acceptor specificity, as well as yielding new disaccharides of the α-series, which are not easily prepared by chemical means.

Preparation and Properties of a Glycosylxylose.

A digest containing phenyl α-glucoside, xylose and enzyme extract was incubated for a period of time, long enough for a sufficient quantity of the glucose-xylose disaccharide to be formed without the formation of too much isomaltose (see Figure 19 facing p.137). The glucose-xylose disaccharide was separated from glucose, phenyl α-glucoside and traces of isomaltose and other impurities by charcoal and preparative paper chromatography. During this procedure very small traces of a mixture of sugars (R_G-value 0.83) which gave a pink

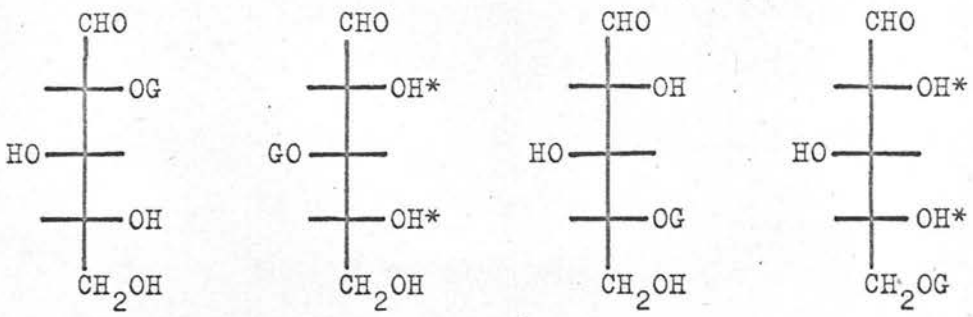
colour with aniline oxalate were isolated. Electrophoresis in borate buffer indicated that the major component of the trace sugars might be a (1→2)-linked glucosylxylose since it had an M_G-value of 0.28 and gave a bright orange fluorescence with aniline oxalate under ultra-violet light.

On treatment of a concentrated solution of the major component with methanol, a white solid crystallised out overnight. The material was washed with ether and dried under vacuum at room temperature to yield 231 mg. The disaccharide gave glucose and xylose in approximately equimolecular amounts on acid hydrolysis, and on reduction followed by hydrolysis glucose and xylitol were formed, indicating that the xylose residue is in the reducing position. The high positive specific rotation $[\alpha]_D^{16}$ + 97° (c 0.89, water) and the lack of hydrolysis by a β-glucosidase preparation from almond emulsin indicated that the compound is α-D-glucosylxylose. Analogous experiments on a disaccharide (the structure of which has been determined in the present investigation as 4-O-β-D-glucopyranosyl-D-xylopyranose) gave $[\alpha]_D^{16}$ -7° (c 1.0, water) and was completely hydrolysed by the β-glucosidase preparation. In all other respects the two glucosylxyloses were identical (see Table 5).

ELECTROPHORESIS OF SUBSTITUTED XYLOSES AND XYLITOLS

(Theoretical mobilities)

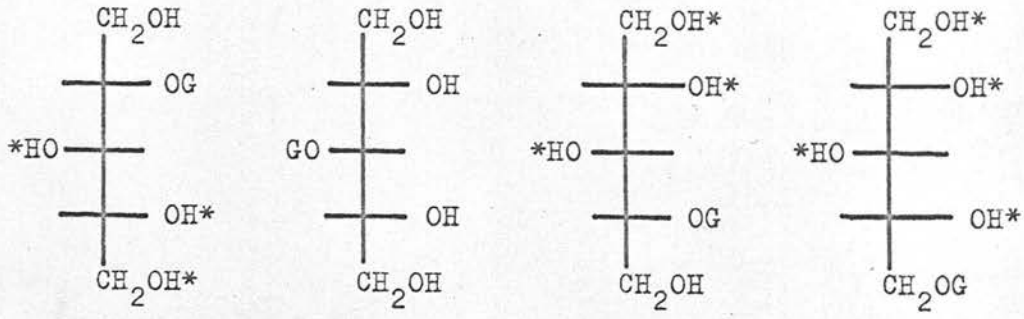
1. Borate buffer:



Linkage	1→2	1→3	1→4	1→5
Mobility	slow	fast	slow	fast

M_G and M_S values:	
"immobile"----	0.00-0.06
"slow"--------	0.15-0.30
"fast"--------	0.50-0.90

2. Molybdate buffer:



Linkage	1→2	1→3	1→4	1→5
Mobility	slow	immobile	slow	fast

*Indicates hydroxyl group involved in complexing.

FIGURE 26[a]

TABLE 5.

Properties of Two Glucosylxyloses.

	Compound I	Compound II
1. R_G-value (10:4:3)	0.68	0.68
2. Colour with aniline oxalate	pink	pink
3. Products on reduction followed by hydrolysis	glucose, xylitol.	glucose, xylitol.
4. Action of almond emulsin, β-glucosidase	-	+
5. $[\alpha]_D^{16}$	+97°	-7°
6. Reaction with triphenyl tetrazolium chloride	+	+
7. Colour with p-rosaniline reagent	blue	blue
8. M_G-value (borate)	0.28	0.28
9. M_S-value on reduced compound (molybdate)	0.30	0.30
10. Periodate consumption mol. prop.	3.86	3.76
11. Sugars on methylation analysis	2,3,4,6-tetra-O-methyl-D-glucose. and 2,3-di-o-methyl-D-xylose.	

Compound I is the glucosylxylose prepared in the present studies. Compound II was obtained by transfer of glucosyl radicals from salicin to xylose by a barley β-glucosidase preparation.

Tentative evidence that the linkage was (1→4) or (1→5) was obtained from the M_G-value of 0.28 in borate buffer, the blue colour with p-rosaniline reagent (see Figure 25) and the pink colour with triphenyl tetrazolium chloride. This was supported by the periodate consumption

DEGRATION SCHEME FOR DISTINGUISHING BETWEEN

TWO POSSIBLE STRUCTURES FOR α-GLUCOSYLXYLOSE.

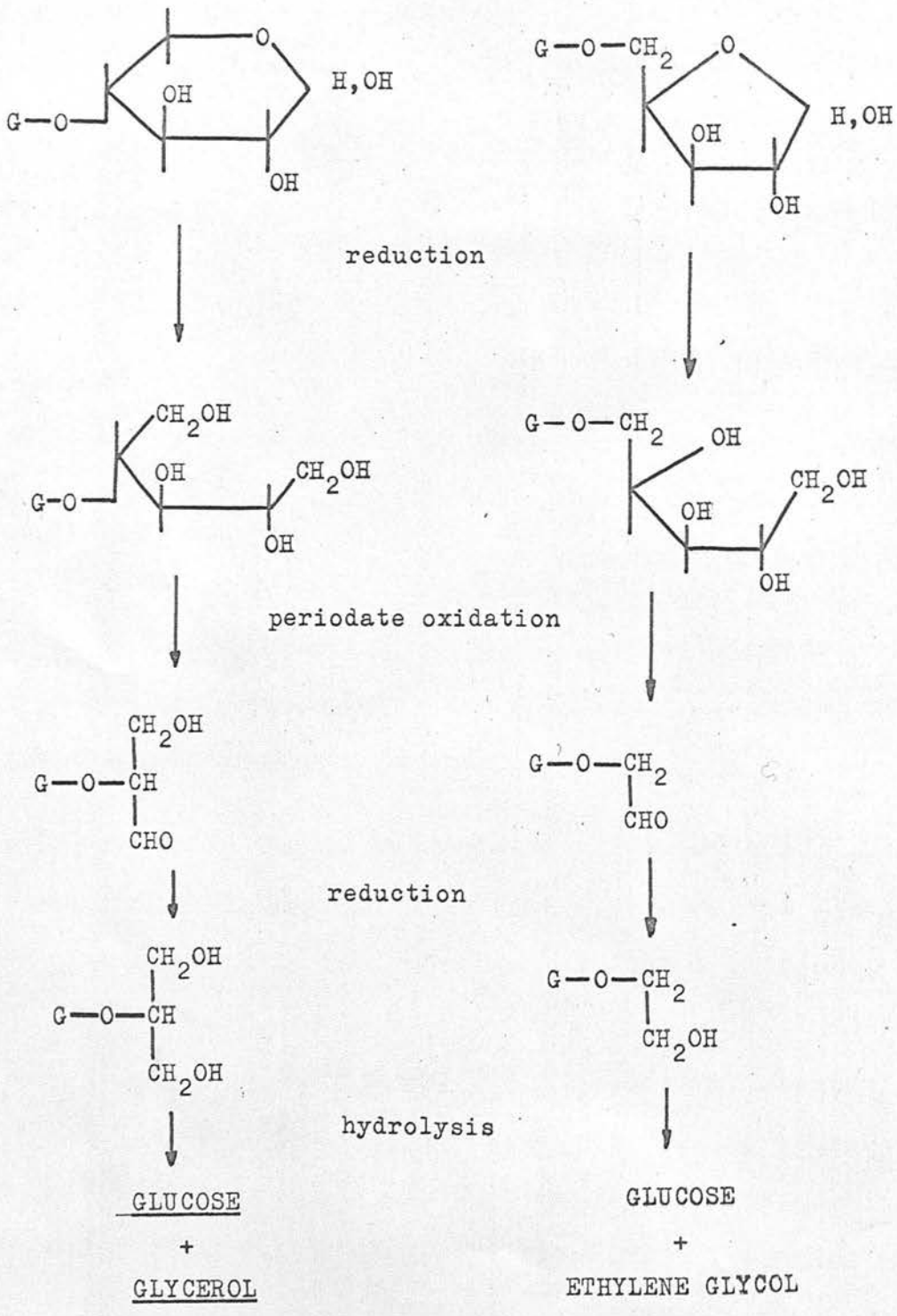


FIGURE 24

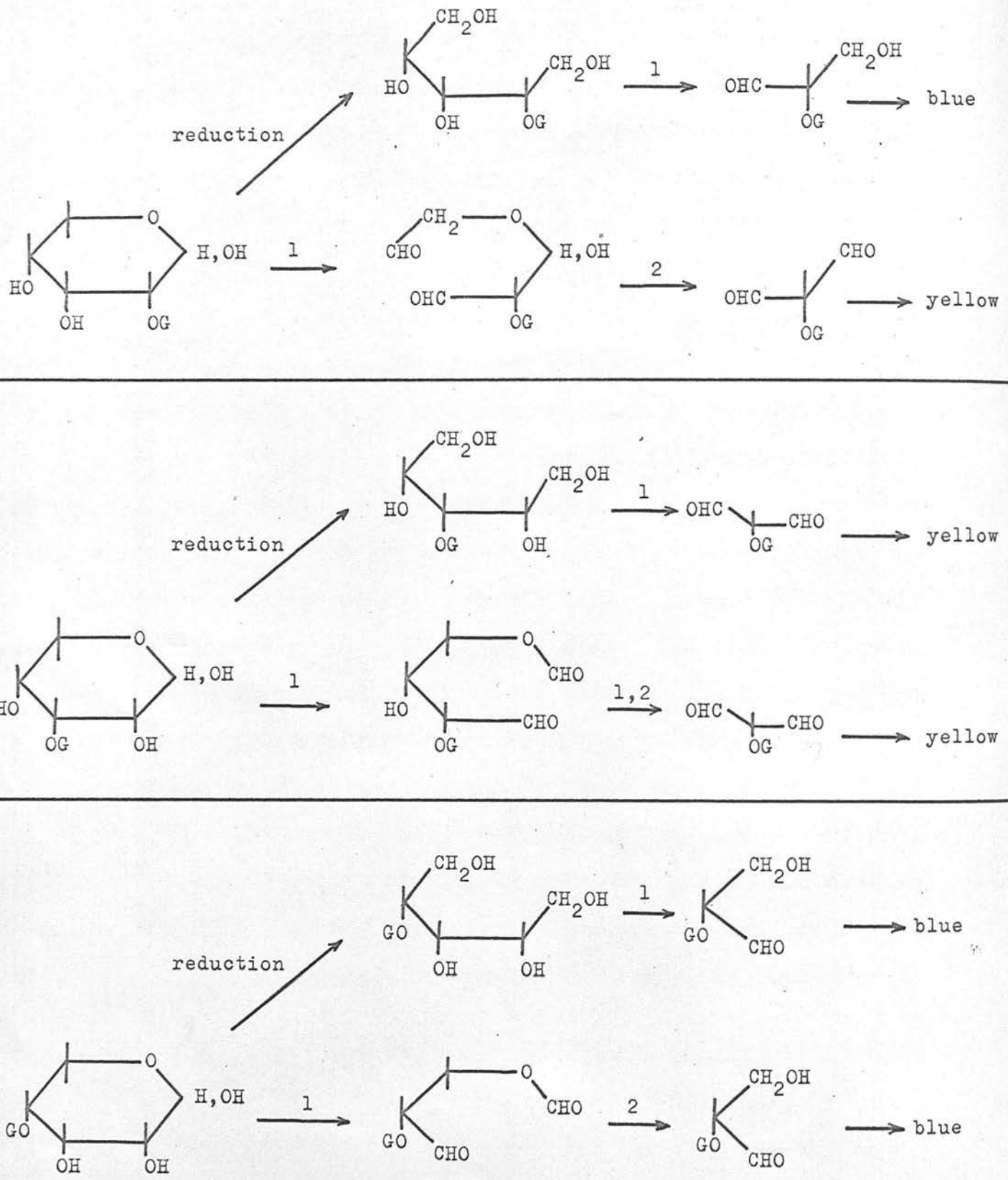
value of 3.9 mol. prop. and the isolation and characterisation of 2,3,4,6-tetra-O-methyl-D-glucose and 2,3-di-O-methyl-D-xylose obtained on methylation analysis. Although xylose is present in the pyranose form and the C-5 hydroxyl group would probably not be available as an acceptor site, it was considered that some attempt should be made to distinguish between the two possibilities. Reduction of a (1→5)-linked glucosylxylose would produce a xylitol moiety with 4 hydroxyl groups in a row and the expected M_S-value would be 0.4-0.9, whereas a (1→4)-linked glucosylxylose would produce, on reduction, a xylitol moiety with 3 hydroxyl groups, those on C-2 and C-3 having an α-trans relationship and the expected M_S-value would be about 0.2-0.3. The experimental value of 0.30 suggested that the linkage was (1→4). Confirmation of this was obtained by the scheme shown in Figure 24. Reduction followed by mild periodate oxidation and reduction will yield a substituted glycerol or a substituted glycol depending on whether the glucosyl substituent (G) is attached to a primary or secondary hydroxyl group in the original material. To test the scheme, maltose and isomaltose were used. Maltose yielded glucose and glycerol, whereas isomaltose produced glucose and glycol. The glucosylxylose yielded glucose and glycerol, thus confirming that the compound is 4-O-α-D-glucopyranosyl-D-xylose.

Preparation and Properties of a Glucosylribose.

The main disaccharide component of a digest containing phenyl α-glucoside, D-ribose and extract I was isolated by methods similar to those used for the preparation of glucosylxylose. However, the

REACTION OF PERIODATE- p-ROSANILINE REAGENT WITH

GLUCOSYLPENTOSES AND GLUCOSYLPENTITOLS



1. Periodate oxidation.
2. Hydrolysis.

FIGURE 25

compound did not crystallise and was obtained as a dry amorphous powder (200 mg.). Trace components (R_G-value 0.85) were also detected. It is thought that the main component of the minor products was a 2-O-glucosylribose since it gave a yellow colour with p-rosaniline before reduction and a blue colour after reduction (see Figure 25) an orange fluorescence with aniline oxalate under ultraviolet light; in addition it gave no colouration with triphenyltetrazolium chloride. The major component gave glucose and ribose on hydrolysis and glucose and ribitol on reduction followed by hydrolysis; it was unattacked by a β-glucosidase preparation from almond emulsin and had a specific rotation $[\alpha]_D^{16}$ + 93°. The compound is therefore an α-D-glucopyranosyl-D-ribose. The rich pink colour with triphenyl tetrazolium chloride reagent showed the absence of a (1→2)-linkage. The blue colour with periodate-p-rosaniline reagent both before and after reduction indicated that the linkage was probably (1→4) or (1→5). The electrophoretic mobility of the reduced compound in molybdate buffer (M_S 0.01) ruled out the possibility of a (1→5)-linkage. Confirmation that the linkage is (1→4) was obtained from the periodate consumption of 3.72 mol. prop. (cf. theoretical value of 4.00). A (1→3) or a (1→2)-linked glucosyl pentose would reduce 3.00 mol. prop. of periodate. Furthermore, the reduced disaccharide had R_G-values (10:4:3 and 18:3:1:4 as solvents), M_G-value (borate buffer) and M_S-value (molybdate buffer) identical with those of an authentic sample of 4-O-α-D-glucopyranosyl-D-ribitol (provided by Dr. A.R. Archibald).

Preparation and Properties of Glucosylarabinoses.

A mixture containing glucose-arabinose disaccharides (total yield 190 mg.) was isolated from a digest containing phenyl α-glucoside, D-arabinose and extract I, by charcoal and thick paper chromatography. Paper chromatography of the mixture in 10:4:3 as solvent and 36hr. development indicated the presence of four components; the R_G-values were component 1, 0.61; component 2, 0.70; component 3, 0.77; component 4, 0.89. All four gave pink colours with aniline oxalate, but in addition component 3 gave a bright orange fluorescence with aniline oxalate under ultraviolet light. It was at first thought that this peculiar fluorescence with aniline oxalate might be due to the presence of an arabinofuranose residue, but an authentic sample of the (1→5)-linked glucosylarabinose which must have the arabinose residue in the furanose form gave no fluorescence. It is therefore thought that this peculiar fluorescence is due to the presence of a 2-O-substituted pentose residue. Furthermore, component 3 gave a yellow colour with the periodate-p-rosaniline reagent and did not react with triphenyl tetrazolium chloride reagent (the other components gave a positive reaction with this latter reagent).

Components 1, 2 and 4 gave blue, yellow and blue colours respectively with periodate-p-rosaniline reagent (see Figure 25 facing p.175). Component 3 had an R_G-value identical with that of 3-O-α-D-glucopyranosyl-D-arabinose and component 4 corresponded to 5-O-α-D-glucopyranosyl-D-arabinose. This preliminary survey suggests that the linkages might be as follows: component 1, (1→4); component 2, (1→3); component 3, (1→2); component 4 (1→5).

The mixture was partially fractionated by thick paper chromatography into fraction A (components 1, 2 and 3) and fraction B (components 3 and 4). The small scale methylation technique of Kuhn, Trischmann and Löw (1955) was applied to each fraction. The products were methanolysed and examined by gas-liquid chromatography. The presence of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3-di-O-methyl-D-arabinose and 3,4-di-O-methyl-D-arabinose was indicated. Peaks corresponding to the methyl glycosides of the 2,4- or 2,5-di-O-methyl-arabinoses would probably overlap with the other products. In fraction A, it therefore seems likely that the 2,3-di-O-methyl-arabinose is produced from the (1→4)-linked glucosylarabinose and the 3,4-isomer from the (1→2)-linked glucosylarabinose, in which case the latter component must have the arabinose residue in the pyranose form. In fraction B the presence of 2,3-di-O-methylarabinose on methylation analysis is consistent with component 4 being a (1→5)-linked glucosylarabinose.

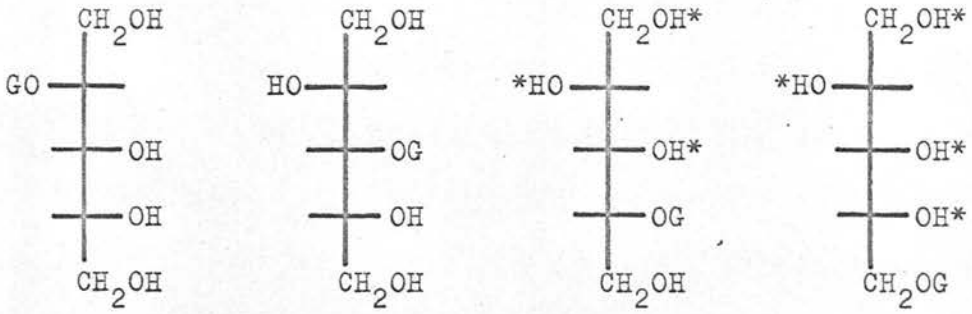
Small amounts of each component were obtained chromatographically pure by successive thick paper separations. The components each gave glucose and arabinose on hydrolysis and on reduction followed by hydrolysis, glucose and arabinitol were produced. The colours formed with the periodate-p-rosaniline reagent before and after reduction were consistent with the tentative structures assigned to each compound (see Fig.25 facing p.175). This technique does not, however, distinguish between the (1→4) and the (1→5) isomers. The reduced disaccharides were examined by molybdate electrophoresis. Component

ELECTROPHORESIS OF SUBSTITUTED ARABINITOLS AND LYXITOLS

(Theoretical mobilities)

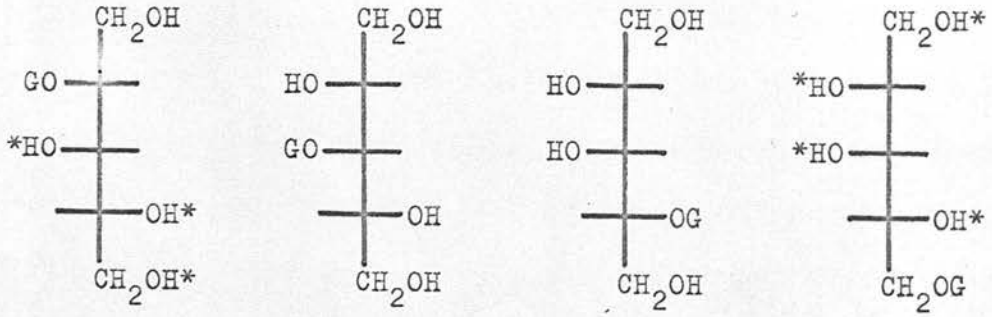
Molybdate buffer pH 5

ARABINITOLS



Linkage	1→2	1→3	1→4	1→5
Mobility	immobile	immobile	slow	fast

LYXITOLS



Linkage	1→2	1→3	1→4	1→5
Mobility	slow	immobile	immobile	fast

*Indicates hydroxyl group involved in complexing.

FIGURE 26[b]

2 was virtually immobile (as was the standard 3-O-α-D-glucopyranosyl-D-arabinitol. Component 4 had an M_S-value of 0.4 (cf. M_S-value of 0.4 for the 5-O-α-D-glucopyranosyl-D-arabinitol. Components 1 and 3 had mobilities within the range postulated by theory (see Figure 26[b]).

It is therefore concluded that transfer of glucosyl radicals by cell-free extracts from Tetrahymena pyriformis occurs to positions 2,3,4 and 5 of D-arabinose resulting in the formation of 2-O-α-D-glucopyranosyl-D-arabinopyranose, 3-O-α-D-glucopyranosyl-D-arabinopyranose, 4-O-α-D-glucopyranosyl-D-arabinose and 5-O-α-D-glucopyranosyl-D-arabinose.

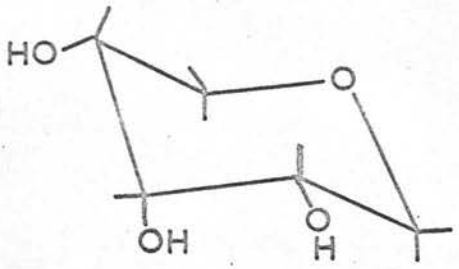
Transfer of Glucosyl Radicals to D-Lyxose.

From a digest containing phenyl α-glucoside, D-lyxose and extract 2, three glucosyllyxoses have been obtained in chromatographically and electrophoretically pure forms. The disaccharides had high positive specific rotations and were unattacked by a β-glucosidase preparation, showing the presence of α-glucosidic linkages. The mode of linkage was investigated by spot tests with triphenyl tetrazolium chloride, periodate-p-rosaniline (see Figure 25 facing p.175) and by molybdate electrophoresis on the reduced disaccharides (see Figure 26[b] facing p.178). The results of these preliminary tests indicated that disaccharide (a) was 4-O-α-D-glucopyranosyl-D-lyxose; disaccharide (b) 2-O-α-D-glucopyranosyl-D-lyxose and disaccharide (c) 3-O-α-D-glucopyranosyl-D-lyxose.

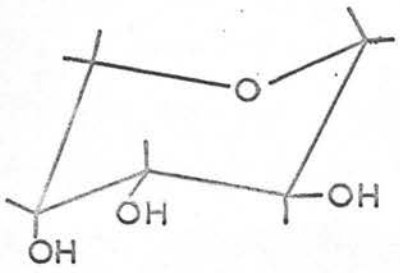
Methylation analysis of disaccharide (a) produced 2,3-di-O-methyl-

D-lyxose and 2,3,4,6-tetra-O-methyl-D-glucose (identified by paper chromatography and gas-liquid chromatography; authentic standards were available). Methylation of disaccharide (b) and disaccharide (c) followed by acid hydrolysis and examination by paper chromatography indicated, in addition to tetramethylglucose, spots which gave pink colours with aniline oxalate. It has been found that all methylated sugars with a hydroxyl group on C-4 give brown colours with aniline oxalate, while those with a methoxyl group on C-4 give pink colours. It therefore seems likely that disaccharides (b) and (c) have the lyxose residue in the pyranose form. The dimethyllyxoses were isolated and reduced. The (1→4) and (1→2)-linked disaccharides would produce compounds which would be susceptible to periodate oxidation but the product from the (1→3)-linked disaccharide would only be susceptible to periodate oxidation if the lyxose residue existed in the furanose form in the original material. By experiment the products from the (1→4) and (1→2)-linked compounds were attacked by periodate but the (1→3)-linked compound was not. This is consistent with disaccharide (a) being 4-O-α-D-glucopyranosyl-D-lyxose and disaccharide (b) being 2-O-α-D-glucopyranosyl-D-lyxopyranose (although the assignment of the lyxose residue as lyxopyranose in this latter compound is only very tentative). However, the resistance of the product from the (1→3)-linked disaccharide confirms the mode of linkage and gives additional evidence that the lyxose residue is in the pyranose form.

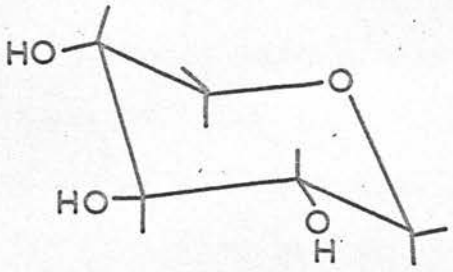
PREFERRED CONFORMATIONS OF THE ALDOPENTOSES



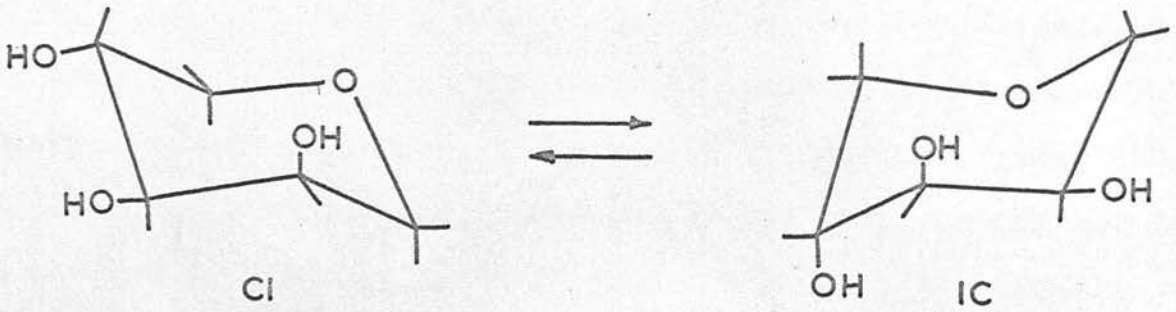
D-Ribose



D-Arabinose



D-Xylose



D-Lyxose

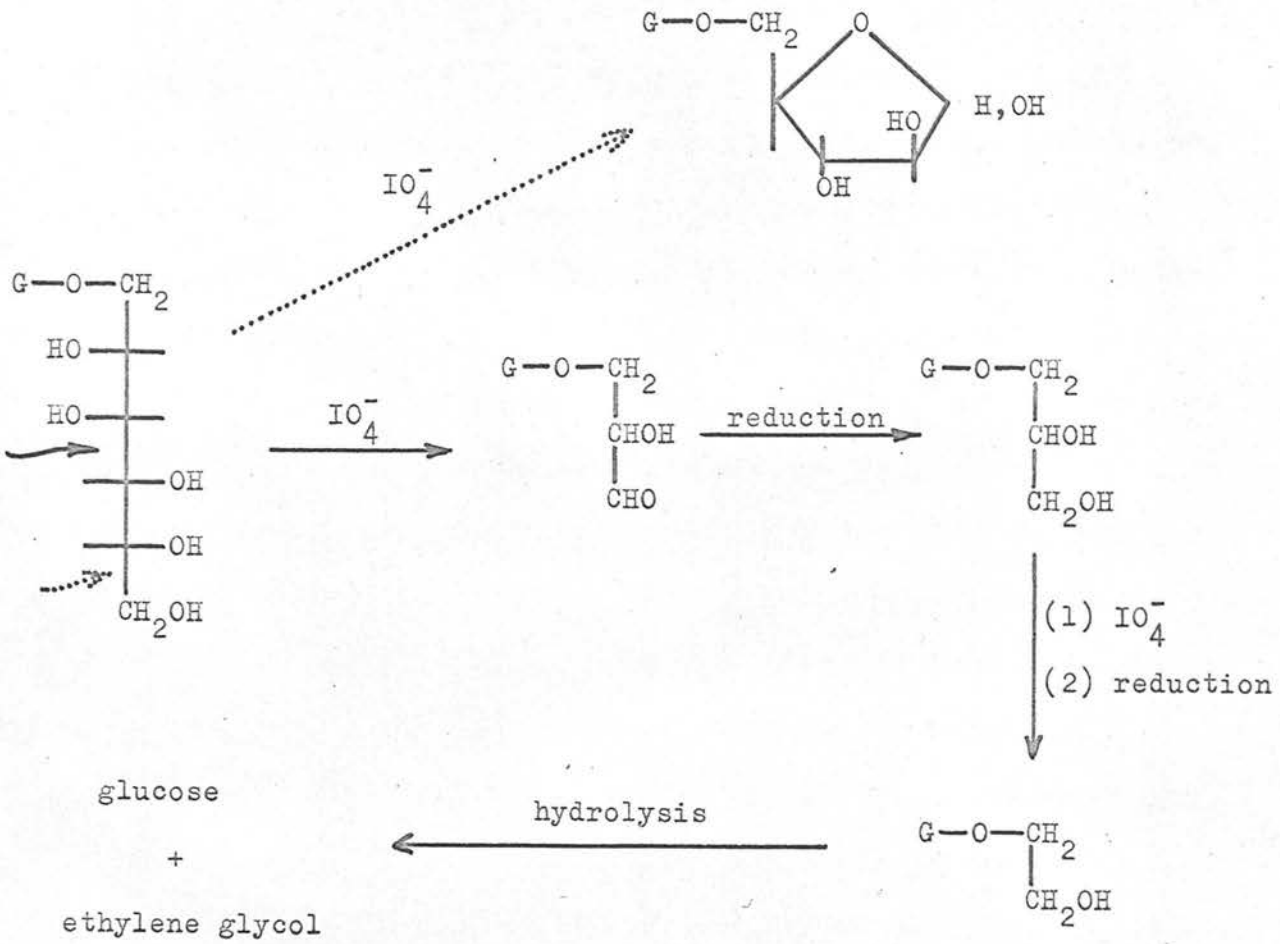
FIGURE 27

Relationship between stereochemistry of the pentoses and transfer products.

The most favourable acceptor site, when glucosyl radicals are transferred to glucose, is the primary hydroxyl group on C-6; the other favourable acceptor site is the hydroxyl group on C-4. This latter conclusion is verified by the fact that 4-O-α-D-glucopyranosyl-D-xylose is formed almost exclusively when glucosyl radicals are transferred to D-xylose; D-xylose being stereochemically identical with D-glucose except that the former compound has no CH_2OH group. Transfer of glucosyl radicals to D-ribose also occurs to the C-4 hydroxyl group. However, with D-lyxose or D-arabinose as acceptors, other positions can also act as acceptor sites.

A probable explanation of these differences can be obtained from stereochemical considerations. Figure 27 shows the preferred conformations of the pentoses. Transfer of glucosyl radicals is unlikely to occur readily to an axial hydroxyl group due to the close proximity of other parts of the pentose molecule, whereas an equatorial hydroxyl group is readily accessible. Thus in D-xylose and D-ribose the C-4 hydroxyl groups are equatorial and transfer occurs almost exclusively to this position. With D-lyxose, which exists as an equilibrium mixture of the C1 and 1C conformations, the C-4 hydroxyl group can be in an equatorial or an axial position. This appears to influence the transferase specificity of the enzyme system and although transfer to the C-4 hydroxyl group still predominates, transfer also occurs in substantial amounts to the C-3 and C-2 hydroxyl groups. With D-arabinose the C-4 hydroxyl group is in the axial position and transfer occurs to the hydroxyl groups on C-2, C-3, C-4 and C-5; transfer to the C-4 hydroxyl group does not predominate.

PERIODATE DEGRADATION OF SUGAR ALCOHOLS UNDER DILUTE CONDITIONS



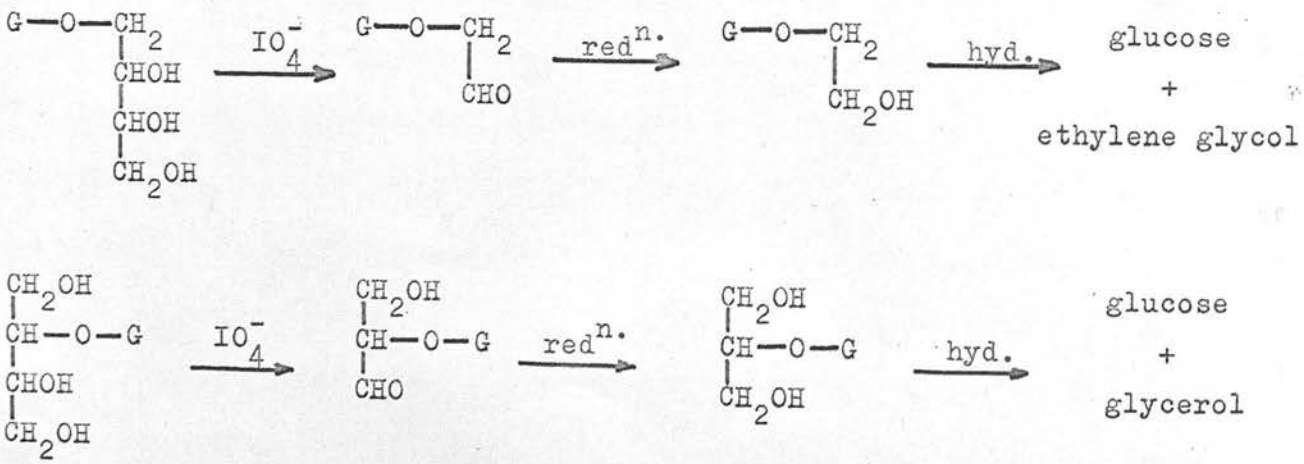


FIGURE 28

The isolation of 5-O-α-D-glucopyranosyl-D-arabinose indicates that arabinofuranose acts as an acceptor of glucosyl radicals.

Transfer of Glucosyl Radicals to Erthritol and Mannitol.

The present experiments indicate that transfer of glucosyl radicals to erythritol and mannitol occurs to the primary hydroxyl groups in each case. The evidence for this is illustrated in Figure 28. Periodate oxidation followed by reduction of glucosylalcohols linked through a secondary hydroxyl group would yield 2-O-α-D-glucosyl -D-glycerol which would be resistant to further periodate oxidation under dilute conditions (cf. Clancy and Whelan, 1959). The isolation of glucosylglycol indicates that the glycosyl radical is attached to a primary hydroxyl group. The present synthesis of 1-O-α-glycopyranosyl-D-mannitol is of interest since it is the α-anomer of the glucosyl-mannitol obtained by partial acid hydrolysis of laminarin.

Transfer Glucosyl Radicals to Mannose and Galactose.

Galactose.

Preliminary studies indicate that transfer of glucosyl radicals occurs to the C-6 hydroxyl groups of mannose and galactose.

Trans β-Glucosylase Activity.

In a preliminary investigation, the sugars produced by incubation of cellobiose with the enzyme extract have been tentatively identified as glucose, laminaribiose, sophorose, gentiobiose and 6^2-β-glucosyl-cellobiose; other products were also present. It is therefore concluded that the trans-β-glucosylase activity possesses a group rather than an absolute activity for the acceptor site and is thus similar to trans β-glucosylase systems from other sources (see for example,

Hutson, 1964). Figure 21 indicates that the transfer products synthesised may vary depending on the extent to which the reaction is allowed to proceed. Preliminary experiments also show that D-xylose can act as an acceptor of β-glucosyl radicals.

Comparison of the Maltase and Phenyl α-Glucosidase Activities.

The difference in the pH optima of the maltase and phenyl α-glucosidase activities is not great enough to distinguish whether the two activities are due to the same enzyme or to two different enzymes. However, heat deactivation experiments suggest that the activities are due to different enzymes; confirmation of this would require fractionation studies on the enzyme extract.

The present experiments provide information on the acceptor specificity of the trans α-glucosylase activity of a cell-free extract from Tetrahymena pyriformis and illustrate how the specificity may be influenced by steric effects. In addition the transferase reactions examined in the present studies provide a route to new α-glucosylpentoses which would be difficult to synthesis by chemical means.

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THE ENZYMIC SYNTHESIS OF SOME GLUCOSYL-PENTOSE DISACCHARIDES.

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Department of Chemistry, The University, Edinburgh 9, Scotland. Previous studies (Archibald and Manners, Biochem. J., 73, 292, 1959) have shown that extracts of the ciliate Tetrahymena pyriformis catalyse the transfer of α-glucosyl radicals from maltose to various mono-, di- and trisaccharides. We now report that phenyl α-D-glucoside can also serve as a glucosyl donor, so that incubation of the extract with a mixture of this glycoside and a pentose leads to the formation of a glucosyl-pentose disaccharide. By this method, various sugars including glucosyl-D-arabinose, glucosyl-D-lyxose, glucosyl-D-ribose and glucosyl-D-xylose have been prepared. The latter has been characterised as O-α-D-glucopyranosyl-(1→4)-D-xylopyranose. From a digest of D-xylose (5 g.) and phenyl α-D-glucoside (4.5 g.) incubated with extract at pH 6 and 37° for 6 days, a mixture of three disaccharides was isolated by charcoal-Celite and preparative paper chromatography. The major product (230 mg.) had $[\alpha]_D$ +97° in water; acid hydrolysates of the disaccharide, and the borohydride-reduced disaccharide contained glucose and xylose, and glucose and xylitol respectively. The disaccharide was not hydrolysed by almond β-glucosidase, and the paper electrophoretic mobility was consistent with the presence of a (1→4)-linkage. This was confirmed by methylation and periodate oxidation studies. The minor products from the digest were traces of a second glucosylxylose and isomaltose. Incubation of the extract with glucose, or xylose, or a mixture of both, did not result in the formation of any disaccharides.

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The Molecular Structure of a Reserve Polysaccharide from *Entodinium caudatum*

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Rumen fluid containing a limited number of ciliate species, i.e. *Entodinium caudatum*, *E. simplex* and *Dasytricha ruminantium*, was obtained from the isolated sheep used by Abou Akkada & Howard (1960). The procedure for the separation of *E. caudatum* containing storage polysaccharide but relatively small amounts of ingested vegetable starch was basically that of Abou Akkada & Howard (1960). A suspension of *E. caudatum* and *D. ruminantium* was obtained by separation and decantation and these organisms were kept for 12 hr. in buffer containing chloramphenicol (50μg./ml.). Treatment for a further 3 hr. in buffer containing 0·6 % mannose killed all the *D. ruminantium*. The *E. caudatum* were then washed and stored at $-20°$ until required.

In preliminary experiments, a cell suspension was disrupted by ultrasonic vibrations, and the defatted cell debris then extracted with dimethyl sulphoxide for 6 days at room temperature (cf. Leach & Schoch, 1962). The extract contained polysaccharide (fraction I) which was precipitated by butanol, and a second fraction, precipitated by ethanol. The fractions resembled amylopectin in being polymers of D-glucose which gave a purple coloration with iodine, and in being partly degraded by both α- and β-amylase. A partial acid hydrolysate of fraction I (glucose content, 84 %; β-amylolysis limit, 59 %) contained glucose, maltose and higher maltosaccharides. Fraction II, on further purification, had a glucose content of 86 %, stained purple with iodine ($\lambda_{max.}$ 540 mμ; blue value 0·10) and by potentiometric titration had an iodine affinity of only 0·38 % (cf. about 4·0 and 19·0 % for a normal plant starch and amylose respectively). The remainder of these fractions was combined and further purified to give fraction III which had a glucose content of 95 %, and $[\alpha]_D + 208°$ (in water). Methylation and acid hydrolysis gave 2,3,6-tri-*O*-methyl-D-glucose as the major product, confirming the presence of $(1 \rightarrow 4)$-linked glucose residues.

A second preparation of fraction III was made. It had a glucose content of 96 %, stained purple with iodine ($\lambda_{max.}$, 540 mμ, blue value 0·10) and had an iodine affinity of 0·30 %. The polysaccharide had a β-amylolysis limit of 58 %, and an average chain length of 19 glucose residues (determined by α-amylolysis of the β-dextrin, cf. Manners & Wright, 1962). These properties are similar to those of plant amylopectins.

We therefore conclude that the reserve polysaccharide of *E. caudatum*, which amounts to 6–7 % of the dry weight, is an amylopectin, similar to those from other holotrich ciliates (cf. Forsyth & Hirst, 1953; Mould & Thomas, 1958).

We wish to thank Professor E. L. Hirst, C.B.E., F.R.S., and Dr P. N. Hobson for their interest in this work.

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Studies on the Metabolism of the Protozoa

10. THE MOLECULAR STRUCTURE OF THE RESERVE POLYSACCHARIDES FROM *OCHROMONAS MALHAMENSIS* AND *PERANEMA TRICHOPHORUM**

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Although several protozoa synthesize iodophilic reserve polysaccharides of the glycogen, amylopectin or starch type (see, for example, Manners & Ryley, 1955; Archibald, Hirst, Manners & Ryley, 1960), others contain iodophobic reserve substances. We now describe the structural analysis of such materials from the flagellated protozoa *Ochromonas malhamensis* and *Peranema tricho-*

* Part 9: Ryley (1962).

phorum. The former is referred to as 'leucosin', even though this term has been incorrectly applied to the starch-type polysaccharide from *Chilomonas paramecium* (Hutner & Provasoli, 1951), and the latter as 'paramylon' (also known as paramylum).

O. malhamensis is a freshwater organism with two unequal flagellae, belonging to the order Chrysomonadina, a group whose members characteristically store oil and leucosin; the former occurs as droplets scattered throughout the cytoplasm, and leucosin accumulates in a posterior vacuole, which may be so large as to almost fill the cell. The organism, about $6\mu \times 10\mu$ in size, is phagotrophic, i.e. can ingest particulate food, and, although it has a golden-brown chromatophore, it can only partially meet its needs by photosynthesis. In pure cultures, carbon requirements are satisfied by sugars, but not by acetate. *P. trichophorum* is a colourless member of the order Euglenoidina. It lives in ponds rich in organic matter, and feeds phagotrophically on a variety of plant and animal food, e.g. other protozoa such as *Ochromonas*. It is an elongate organism, approximately $55\mu \times 22\mu$ in size in culture, that can move either by swimming or gliding; it has a stout flagellum about as long as the body projecting anteriorally, and another more delicate flagellum extending spirally backwards adherent to the pellicle. Paramylon is stored in the form of flat granules about $3{\cdot}4\mu$ diam. $\times$ $2{\cdot}3\mu$ thick. Excellent descriptions of the structure and biology of these two organisms are given by Pringsheim (1952) and Chen (1950).

In preliminary accounts of this work (Archibald, Manners & Ryley, 1958; Cunningham, Manners & Ryley, 1961), evidence for the presence of β-(1$\rightarrow$3)-glucosidic linkages in both polysaccharides was described. The same type of linkage has also been found in the reserve polysaccharides from the protozoa *Euglena gracilis* (Clarke & Stone, 1960) and *Astasia ocellata* (D. J. Manners, J. F. Ryley & J. R. Stark, unpublished work).

MATERIALS AND METHODS

Materials

Ochromonas malhamensis was grown in daylight (not essential) at 28° in a medium containing 0·1% of Oxoid peptone, 0·1% of Hepamino liver extract (Evans Medical Ltd., Speke, Liverpool 24) and 0·1% of glucose at pH 6·0; 2 l. flasks containing 1·5 l. of medium were used for bulk growth. Cells from 15 l. of culture fluid were harvested by centrifuging after 6 days of growth, suspended in 120 ml. of culture fluid, heated at 100° for 10 min. and centrifuged while hot. The residue was twice extracted with 150 ml. of water at 100° for 10 min. and centrifuged while still hot. The three extracts were treated with ethanol (2 vol.) and allowed to stand overnight. The precipitates from six such batches of medium were combined and used for leucosin preparations I and II.

From a further 75 l. of culture fluid, polysaccharide material was obtained in a similar way, and from it leucosin preparation III was isolated.

Peranema trichophorum was grown in daylight (not essential) at 20° in a medium containing 10% (v/v) of soil extract and 2% (v/v) of milk at pH 7·0. The soil extract was prepared by autoclaving garden soil with an equal weight of water at 15 lb./in.^{2} for 20 min., allowing it to settle and centrifuging the supernatant to clarify it. The complete medium was sterilized by steaming for 30 min. on 3 consecutive days for small quantities, but for bulk growth 2 l. conical flasks containing 1·5 l. of 10% (v/v) soil extract were sterilized by autoclaving, and the required quantity (30 ml.) of milk, previously sterilized by steaming, was added together with the inoculum. Stock cultures were carried in 10 ml. quantities of medium in tubes, being subcultured every 2 weeks. For bulk growth, 250 ml. amounts of medium were inoculated with 1 tube of culture, and, 2 weeks later, one such flask was used to inoculate a 2 l. flask. Organisms were grown in 54 l. batches of medium, harvested by centrifuging after 4 weeks of growth, and washed and stored in methanol, or freeze-dried.

Methods

Analytical methods. The analytical methods employed were those described by Manners & Ryley (1952, 1955), with the following exceptions. Paper chromatograms were developed with ethyl acetate–pyridine–water (10:4:3, by vol.) (solvent 1), butan-1-ol–pyridine–water (6:4:3, by vol.) (solvent 2) or butan-2-one–acetic acid–water, saturated with boric acid (9:1:1, by vol.) (solvent 3). Chromatographic mobilities are expressed relative to that of D-glucose (R_G 1·00). The formaldehyde liberated on periodate oxidation was measured with either a phenylhydrazine–ferricyanide reagent (Hough, Powell & Woods, 1956) or a chromotropic acid reagent (MacFadyen, Watkins & Anderson, 1945). The reduction of periodate was determined either volumetrically (Fleury & Lange, 1933) or spectrophotometrically (Aspinall & Ferrier, 1957).

Mannose in the presence of glucose was determined from the decrease in reducing power caused by treatment of the mixed sugars with glucose oxidase. Solutions of glucose (about 20 mg.) and mannose (about 5 mg.) were mixed and incubated at 35° with glucose-oxidase solution (75 mg. of Takamine DeO enzyme preparation in 5 ml. of water) in a total volume of 25 ml. Samples (6 ml.) were removed at intervals, heated (3 min. at 100°), cooled and centrifuged. The reducing-sugar content of 2 ml. portions was determined, and the results showed that 30 hr. incubation was required for the complete oxidation of glucose. When 20·1 mg. of glucose and 2·36 mg. of mannose were analysed, the observed sugar contents were 20·5 mg. of glucose (102% of theoretical) and 2·26 mg. of mannose (96% of theoretical). The results obtained by this method are regarded as being accurate only to $\pm 5\%$.

Preparation of polysaccharide alcohols. Polysaccharide (200 mg.) was dissolved or suspended in water (10 ml.) and potassium borohydride (100 mg.) added. After mechanical shaking for 48 hr., the excess of borohydride was decomposed with acetic acid, ethanol added and the mixture centrifuged. The polysaccharide alcohols were washed with ethanol and ether, and dried at 60° over phosphorus pentoxide. The glucose contents (on which the yield of formaldehyde produced on periodate oxidation is calculated) were

86% for leucositol and 95% for paramylitol, the impurity being inorganic material.

Periodate-oxidation analysis. The general procedure followed was that described by Anderson, Hirst, Manners & Ross (1958); results are expressed throughout as mol.prop./anhydroglucose residue. Oxidation of a linear chain of (1→3)-linked glucose residues at 2° will liberate 1 mol.prop. of formic acid from any non-reducing terminal groups or other triol groups. At the reducing group an intermediary formyl ester is formed, which is hydrolysed only very slowly, so that formic acid and formaldehyde are released after days rather than hours. At room temperature (18–20°) the ester is slowly hydrolysed with the eventual formation of 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde. At this temperature some 'over-oxidation' (see below) is apparent, but the initial production of formic acid can be obtained by extrapolation of the linear portion of the graph of formic acid production against time to zero time.

The degree of polymerization (i.e. the number of glucose residues/molecule) can be calculated from the weight of polysaccharide producing 1 mole of formaldehyde, or the weight of polysaccharide alcohol yielding 2 moles of formaldehyde.

Periodate oxidations were also carried out at pH 8 as described by Hough & Perry (1956). In these experiments a (1→3)-linked glucosan is oxidized, in a stepwise manner from the reducing group, with the liberation of 1 mol.prop. of formaldehyde/glucose residue. The 'over-oxidation' is arrested by (1→6)-linked glucose residues or other structural features that do not lead to the formation of intermediary malonaldehyde derivatives. (Over-oxidation can be regarded as the production of formic acid and formaldehyde, and the reduction of periodate, by oxidative reactions not involving the cleavage of α-glycol groups.)

For the formaldehyde determinations, samples (1 ml.) were withdrawn from the oxidation mixtures and M-sodium sulphite (1 ml.) and ethanol (5 ml.) added to precipitate polysaccharide. Samples (1 ml.) of the supernatant solution were then analysed by the chromotropic acid method. This modification was devised by Parrish (1959) to decrease interference between carbohydrate and sulphuric acid.

RESULTS

Examination of the polysaccharides from Ochromonas malhamensis

Isolation of leucosin preparation I. The crude polysaccharide preparation was extracted with water (1·5%, w/v) for 1 hr., at room temperature, and insoluble material removed by centrifuging. Ethanol (3 vol.) was added to the extract, the precipitate redissolved in water, the solution centrifuged and polysaccharide material reprecipitated with ethanol. The purification was repeated to give a buff material that contained: anhydrohexose (as glucose), 79%; ash, 3·4%; protein, 13%. An acid hydrolysate contained glucose, small amounts of galactose and mannose, and a trace of xylose (by paper chromatography).

The material was partly deproteinized by 12 treatments with 0·1M-sodium chloride and toluene (Anderson & Greenwood, 1955), the protein content being decreased from 13·1 to 5·6%.

A portion of the product (4·0 g.) was dissolved in water (200 ml.), and 3% (w/v) Cetavlon (cetyltrimethylammonium bromide) solution (100 ml.) was added slowly with stirring (cf. Barker, Stacey & Zweifel, 1957). A brown precipitate was removed, and ethanol (3 vol.) was added to the supernatant solution. Cetavlon was removed from the precipitate by a further six ethanol precipitations to yield a white powder (2·3 g.) referred to below as 'leucosin preparation I'.

Analysis of leucosin preparation I. Paper-chromatographic examination of a total acid hydrolysate showed proportions of glucose, mannose and xylose similar to those present before precipitation with Cetavlon, but a decrease in the amount of galactose. Leucosin preparation I had $[\alpha]_D + 15°$ (*c* 0·3 in water), and contained: anhydrohexose (as glucose), 90%; ash, 1·9%; protein, 4·5%.

A sample (10 mg.) was partly hydrolysed by heating at 100° in 0·5N-sulphuric acid for 1·5 hr. Paper chromatography showed the presence of the four monosaccharides, and of sugars having the same mobilities as authentic samples of laminaribiose, -triose and -tetraose, together with a fourth sugar. The R_G values in solvent 2 were: 0·81, 0·56, 0·38 and 0·28; Peat, Whelan & Lawley (1958) quote values of 0·80, 0·57, 0·40 and 0·28 for the laminaribiose, -triose, -tetraose and -pentaose respectively.

Incubation of a sample (10 mg.) with 10 mg. of an endo-β-glucosidase preparation from *Cladophora rupestris* [prepared by Dr W. A. M. Duncan (see Duncan, Manners & Ross, 1956)] in 0·5 ml. of water for 48 hr. resulted in the formation of glucose, mannose, laminari-biose, -triose, -tetraose and higher sugars. The enzyme preparation liberated a similar series of oligosaccharides from laminarin. Incubation with almond emulsin (an exo-β-glucosidase), prepared by Dr F. B. Anderson, gave only a small amount of glucose.

Purification of leucosin preparation I. Attempts to remove the mannose-containing polysaccharide by copper-complexing or by precipitation with Cetavlon at various pH values were not successful. However, small-scale experiments showed that some fractionation occurred on precipitation with acetone.

Leucosin preparation I (2·0 g.) was dissolved in water (700 ml.) containing 0·5 g. of ammonium acetate. Acetone (800 ml.) was added slowly with stirring, and the precipitate (I*a*) was collected by centrifuging, dissolved in water, reprecipitated and dried. Acetone (1000 ml.) was then added to the supernatant solution to yield a second fraction (I*b*), and from the remaining solution a third fraction (I*c*) was obtained after the addition of a further 1000 ml. of acetone. A portion of each fraction was hydrolysed and examined by paper chromatography. The yields of the fractions, and the constituent sugars, were as follows: I*a* (445 mg.), galactose (+++), glucose (++), mannose (++); I*b* (771 mg.), glucose (+++), mannose (+),

galactose (±); Ic (723 mg.), glucose (++), mannose (+), xylose (±).

Fraction Ic was redissolved in water (200 ml.) and precipitated with acetone (600 ml.) to yield a polysaccharide (I*d*) (400 mg.) that was free from xylose.

The glucose and mannose contents were determined by the glucose-oxidase method to be 71 and 11% respectively for fraction 1*b*, and 78 and 11% respectively for fraction 1*d*. The fractions were combined and dialysed for 3 days against distilled water (a control experiment had shown that 98% of the polysaccharide was retained on dialysis). A slight brown precipitate was removed by centrifuging, followed by filtration through an asbestos pad, and ethanol (3 vol.) was then added to give 'leucosin preparation II' (770 mg.).

Analysis of leucosin preparation II. By the glucose-oxidase method, the glucose and mannose contents were 85 and 12% respectively. The reducing power measured against a laminaribiose standard corresponded to the apparent presence of 1 reducing group/63 hexose residues, indicating polysaccharide rather than oligosaccharide material. Leucosin preparation II had $[\alpha]_D + 10{\cdot}8°$ (*c* 1·0 in water).

Leucosin preparation II (249·0 mg.) was dissolved in water (25 ml.). A portion of this solution (20 ml.) was cooled to 2°, 0·3M-sodium metaperiodate solution (4 ml.) was added, and the mixture was diluted to 100 ml. with water. Samples (10 ml.) were withdrawn at intervals for measurement of formic acid, which amounted to 0·058, 0·080 and 0·085 mol.prop. after 2, 7 and 9 days respectively. The average value of the formic acid content after 7–9 days corresponds to 1 molecule/12 hexose residues. The reduction of periodate, from analysis of 5 ml. samples, corresponded to 0·16 and 0·18 mol.prop. after 7 and 9 days respectively. The production of formaldehyde, as measured by the ferricyanide method, was constant at 0·029 mol.prop. between 7 and 9 days, and corresponded to 1 molecule/35 hexose residues.

In a second experiment in which 195·1 mg. of leucosin preparation II was oxidized at 2°, for 6 days, the reduction of periodate was 0·17 mol.prop. and the production of formic acid 0·078 mol.prop. After a further 48 hr. at 20°, these values had increased to only 0·18 and 0·081 mol.prop. respectively. The average formic acid production corresponded to 1 molecule/12·6 hexose residues.

Leucosin preparation II (9·7 mg.) was also oxidized at 35° and pH 8 with sodium metaperiodate. [The reagents were 0·3M-sodium metaperiodate (2 ml.), 0·1M-sodium phosphate buffer (12 ml.) and water to 25 ml.] The production of formaldehyde was constant after 18 hr. (analysis was continued for 4 days), and amounted to 0·53 mol.prop.

Isolation of leucosin preparation III. A second sample of crude polysaccharide preparation was extracted with water, precipitated with ethanol, and treated with Cetavlon (as described above), to yield a white powder (5·6 g.). This was dissolved in water (1 l.) containing ammonium acetate (0·5 g.), and three fractions were obtained by precipitation with acetone, as follows: 1200 ml. of acetone gave fraction A (1·91 g.), a further 1600 ml. of acetone gave fraction B (2·90 g.), and, finally, 3500 ml. of acetone gave fraction C (0·31 g.).

By paper chromatography, fraction A contained glucose (+++), galactose (+) and mannose (±), fraction B contained glucose (+++), galactose (±) and mannose (±), and fraction C contained glucose (++), mannose (+) and xylose (±).

Fraction B (2·0 g.) was dissolved in water (500 ml.), and acetone (750 ml.) was added to give fraction B1 (0·23 g.). The addition of further acetone (1500 ml.) to the supernatant solution gave fraction B2 (1·24 g.). The latter was purified by dialysis, filtration through an asbestos pad and precipitation with ethanol to yield a white powder (1·05 g.), referred to below as 'leucosin preparation III'.

Characterization of the sugars in fraction A. A sample (0·7 g.) was hydrolysed with 2N-sulphuric acid (70 ml.) at 98° for 2 hr., neutralized (with barium carbonate) and the volume decreased to 5 ml. Part of the hydrolysate (4 ml.) was applied to a column of Dowex 50W (X8; 200–400 mesh; Ba^{2+} form) resin and the monosaccharides were eluted with distilled water (Jones & Wall, 1960). Fractions (3–4 ml.) were collected at intervals, and analysed by paper chromatography. Fractions 30–34 contained glucose, 36–40 contained glucose and galactose, 42 contained these sugars and xylose, 44 also contained mannose, and 46–47 contained the three hexoses. Concentration of fractions 29–34 gave D-glucose, characterized as the penta-acetate (m.p. and mixed m.p. 130–131°); from fractions 39–41, D-galactose was obtained as the crystalline 1-methyl-1-phenylhydrazone (m.p. and mixed m.p. 180–183°). Fractions 44–50 were applied to Whatman 3 MM paper, and the D-mannose was separated, eluted and characterized as the phenylhydrazone (m.p. and mixed m.p. 190–193°).

The molar proportion of the three hexoses was determined by the method of Wilson (1959). From paper chromatograms developed with solvent 1, the molar ratio of glucose to galactose was 86·6:13·4, and from chromatograms developed in solvent 3, which give an improved separation of mannose from the other hexoses, the following composition was obtained: glucose, 86%; galactose, 13%; mannose, 1%.

Analysis of leucosin preparation III. The polysaccharide had $[\alpha]_D + 9{\cdot}1°$ (*c* 0·4 in water), and a reducing power equivalent to an apparent degree of polymerization of 63, indicating a polysaccharide rather than an oligosaccharide nature. On hydrolysis with acid, it gave glucose and no other sugar (by paper chromatography). By cuprimetric titration, the glucose content was 91%, and the analytical results are based on this value. Further examination of an acid hydrolysate showed: (*a*) that

the sugar was destroyed by D-glucose oxidase; (*b*) that non-reducing carbohydrates, e.g. mannitol and sorbitol, were absent. Under similar conditions, the presence of mannitol in an acid hydrolysate of laminarin could be readily detected.

Leucosin preparation III (184 mg. in 20 ml. of water) was oxidized with a mixture of 0·3 M-sodium metaperiodate (20 ml.) and 5% (w/v) potassium chloride (15 ml.) at 20°. The production of formic acid after 3, 5, 7 and 9 days amounted to 0·079, 0·091, 0·095 and 0·100 mol.prop. respectively. Extrapolation of the results indicated that, in the initial oxidation, 0·078 mol.prop. was liberated, corresponding to 1 molecule/12·8 glucose residues.

Leucosin preparation III (196 mg.) was also oxidized with 0·3 M-sodium metaperiodate solution (8 ml.) in a total volume of 100 ml. at 20°. The reduction of periodate was 0·15 and 0·17 mol.prop. after 24 and 48 hr. respectively, and the initial production of formic acid corresponded to 1 molecule/12·8 glucose residues.

Oxidation of leucosin preparation III (11·5 mg.) at pH 8 in a total volume of 25 ml. gave the following production of formaldehyde: 0·39, 0·44, 0·50, 0·50 and 0·49 mol.prop. after 21, 25, 30, 72 and 168 hr. respectively. Under similar conditions, laminarin gave 0·54 mol.prop. of formaldehyde.

Leucosin preparation III (40·4 mg.) and leucositol prepared from it (30·2 mg.) were each dissolved in water (9 ml.) and oxidized with 0·3 M-sodium metaperiodate solution (1 ml.) at 17°. The final production of formaldehyde corresponded to degrees of polymerization of about 35 and 33 glucose residues respectively (see Table 1). At 2°, leucosin failed to give a significant amount of formaldehyde within 1 hr., showing the absence of mannitol-terminated chains of glucose residues (cf. Anderson *et al.* 1958).

Leucosin preparation III (20·0 mg.) was dissolved in 0·05 M-sodium citrate buffer, pH 4·8 (5 ml.), and *Rhizopus arrhizus* enzyme preparation (4 mg.) was added. This preparation showed laminarinase but not cellodextrinase activity (see Cunningham & Manners, 1961). The digest was incubated at 37°, when the apparent percentage conversion into glucose after 3, 21, 46 and 96 hr. was 52, 66, 72 and 75 respectively. Paper chromatography (with solvent 2) showed the presence of glucose and laminaribiose, and a trace of an oligosaccharide with R_G 0·53. In a control digest with laminarin, the same sugars were produced and about 71% of the polysaccharide was converted into reducing sugar (as glucose).

The infrared-absorption spectrum of leucosin preparation III was examined in Nujol with a Perkin–Elmer Infracord spectrophotometer. The spectrum showed an absorption band at 890 cm.$^{-1}$, and was almost identical with that of laminarin, and with that of a sample of chrysolaminarin provided by Dr E. E. Percival (cf. Beattie, Hirst & Percival, 1961).

Smith degradation of leucosin preparation III. Leucosin preparation III (99 mg.) was oxidized with 0·3 M-sodium metaperiodate solution (3 ml.) in a total volume of 25 ml. for 48 hr. at 18°. Excess of periodate was destroyed by the addition of 12·6% (w/v) sodium sulphite solution (12·5 ml.), and, after 30 min., potassium borohydride (100 mg.) was added. After 40 hr. at 18°, the excess of borohydride was decomposed by the careful addition of acetic acid to give pH 6–7. The solution was made 0·1 N with respect to sulphuric acid and maintained at 18° for 24 hr. to effect partial hydrolysis of the polyalcohol. (The general conditions are similar to those employed in parallel studies on laminarin by Smith & Unrau, 1959.) The hydrolysate was neutralized with sodium hydroxide solution (to pH 7) and dialysed for 48 hr. against five successive changes of distilled water (3 l. each).

The non-diffusible material was recovered by freeze-drying. The yield was 114 mg.; the glucose content was 79%, equivalent to a 91% recovery of polysaccharide. The impurity was inorganic material.

Undiffusible polysaccharide (36·3 mg., based on glucose content) was oxidized with 2 mM-sodium metaperiodate (5 ml.) at 2° for 24 hr. Samples (1 ml.) were removed at intervals, and treated with 12·6% (w/v) sodium sulphite solution (0·5 ml.) and ethanol (6 ml.) to remove respectively excess of periodate and polysaccharide, and the formaldehyde contents of the supernatant solutions were measured by the chromotropic acid procedure. The production of formaldehyde after 2, 5·5 and 24 hr. amounted to 0·0062, 0·0059 and 0·0058 mol.prop. respectively. A molecule containing only one internal (1→6)-glucosidic linkage would yield about 0·03 mol.prop. of formaldehyde.

Table 1. *Production of formaldehyde by the periodate oxidation of leucosin, paramylon and their respective alcohols*

The oxidation conditions are given in the text. The formaldehyde results are expressed as mol.prop./glucose residue.

Time of oxidation (days)	Leucosin		Leucositol		Paramylon		Paramylitol	
	Formaldehyde release	Degree of polymerization	Formaldehyde release	Degree of polymerization	Formaldehyde release	Degree of polymerization	Formaldehyde release	Degree of polymerization
1	0·0069	145	0·0281	71	0·0031	320	0·0057	370
5	0·0169	59	0·0418	48	0·0062	161	0·0096	208
11	0·0215	47	0·0519	38	0·0097	103	0·0167	121
18	0·0267	37	0·0576	35	0·0128	78	0·0189	106
22	0·0272	37	0·0590	34	0·0130	77	0·0213	94
29	0·0283	35	0·0614	33	0·0142	71	0·0225*	89*

* 0·0228 and 88 after 35 days.

Preparation of leucosin acetate. Fraction B (see above) (500 mg.) was dispersed in formamide (5 ml.), and treated with acetic anhydride (4 ml.) and pyridine (10 ml.). The mixture was shaken in the dark for 4 days and then poured into ice–water (500 ml.) with stirring. The precipitated acetate was washed several times with water, filtered and dried. It was purified by solution in chloroform and precipitation with light petroleum (b.p. 80–100°). The yield was 480 mg.; the acetyl content was 41·2%; $[\alpha]_D$ −59·3° (*c* 0·9 in chloroform).

Examination of the polysaccharide from Peranema trichophorum

Isolation of paramylon. In preliminary experiments, extraction of the cells with water or dilute sodium hydroxide solution gave low yields of an impure glucose-containing polysaccharide. The procedure of Clarke & Stone (1960) was therefore adopted. The cells from 54 l. of culture medium were suspended in water, and disrupted by ultrasonic vibrations. Fatty material was discarded, and the cell debris was washed with alcohol and ether and dried. The yield was 1·8 g. This material was incubated with trypsin (95 mg. of a Nutritional Biochemicals Corp. preparation in 50 ml. of 0·1 M-sodium phosphate buffer, pH 7·6) at 40° for 40 hr., the mixture centrifuged, and the residue extracted twice with saturated urea solution and washed with water. The remaining denatured protein was then removed by shaking the off-white material for 60 min. with chloroform (50 ml.), water (130 ml.) and pentanol (20 ml.), and separating the chloroform–protein gel by centrifuging. Ethanol (3 vol.) was added to the aqueous phase, and the white particles of paramylon were collected, washed with ethanol and then ether, and air-dried. The yield was 1·06 g. (59% of the dried cell material).

Examination of paramylon. Paramylon was insoluble in water, N-sodium hydroxide and 2N-sulphuric acid. For acid hydrolysis 20 mg. was heated with 90% formic acid solution (2 ml.) at 98° for 2 hr., and then 2N-sulphuric acid (4 ml.) was added and heating continued for a further 3 hr. The neutralized hydrolysate contained only glucose, and, by cuprimetric titration, the content was 97%. A partial acid hydrolysate contained glucose and four oligosaccharides with the R_G values of laminarisaccharides. Treatment of this hydrolysate with glucose oxidase caused the destruction of glucose and the formation of gluconic acid (detected by paper chromatography).

Paramylon dissolved slowly in cold 5N-sodium hydroxide solution ($[\alpha]_D$ +16°; *c* 1·2). A suspension (59 mg.) in 15 mM-sodium metaperiodate (10 ml.) was kept at 27° for 72 hr. The reduction of periodate was 0·03 mol.prop., whereas under the same conditions laminarin reduced 0·30 mol.prop.

Paramylon (38·0 mg.) and paramylitol prepared from it (26·7 mg.) were each suspended in water (9 ml.) and oxidized with 0·3 M-sodium metaperiodate solution (1 ml.) at 17°. The results (Table 1) show that the degree of polymerization is about 80 glucose residues. At 2° there was no significant release of formaldehyde from paramylon, indicating a similarity to leucosin and a difference from laminarin. On periodate oxidation at pH 8, the production of formaldehyde after 5, 19, 25 and 30 days was 0·22, 0·31, 0·30, 0·29 mol.prop. respectively.

Paramylon (20 mg.) was dissolved with difficulty in 2N-sodium hydroxide solution, neutralized, and pH 4·8 buffer and *Rhizopus* preparation were added as for leucosin III (see above). After incubation at 37° for 24 and 48 hr., the presence of glucose and laminaribiose was shown by paper chromatography. Since a large part of the paramylon was not in solution, cuprimetric titrations were not carried out. Samples of chrysolaminarin and the glucosan from *Euglena gracilis* (prepared by the method of Clarke & Stone, 1960) on incubation with the *Rhizopus* preparation were rapidly hydrolysed (within 1·5 hr.) to glucose and laminaribiose. Laminaritriose was also present in the chrysolaminarin digest.

In a Nujol mull, the paramylon gave an infrared-absorption spectrum almost identical with that of laminarin, showing an absorption band at 890 cm.$^{-1}$.

DISCUSSION

The present investigation shows that the flagellate *Ochromonas malhamensis* synthesizes a mixture of polysaccharides including polymers of D-glucose, D-galactose and D-mannose. By precipitation with Cetavlon and with acetone a pure glucosan (leucosin) can be obtained, which contains β-glucosidic linkages, as shown by the low specific rotation, hydrolysis by β-glucosidase preparations and the infrared-absorption spectrum. The presence of (1→3)-linkages is indicated by the resistance of the polysaccharide to periodate oxidation, the formation of laminarisaccharides on partial hydrolysis with acid, and the production of glucose and laminaribiose on enzymic degradation.

On periodate oxidation with either sodium metaperiodate at 2° or with potassium metaperiodate at 18–20°, the production of formic acid corresponded to the presence of one 'triol' group (i.e. non-reducing end group, or internal (1→6)-glucosidic linkage) per 12–13 glucose residues. Since the degree of polymerization is approximately 34, the molecules each contain on the average three 'triol' groups which may be present as (*a*) non-reducing end groups, implying the presence of two branch points/molecule, (*b*) two internal (1→6)-glucosidic linkages/molecule or (*c*) one branch point and one internal (1→6)-glucosidic linkage/molecule. Structures of the (*b*) and (*c*) type exist in yeast glucan (Peat, Whelan & Edwards, 1958) and chrysolaminarin (Beattie *et al.* 1961).

It is unfortunate that the solution of this problem by methylation or partial acid hydrolysis requires larger quantities of leucosin than were available.

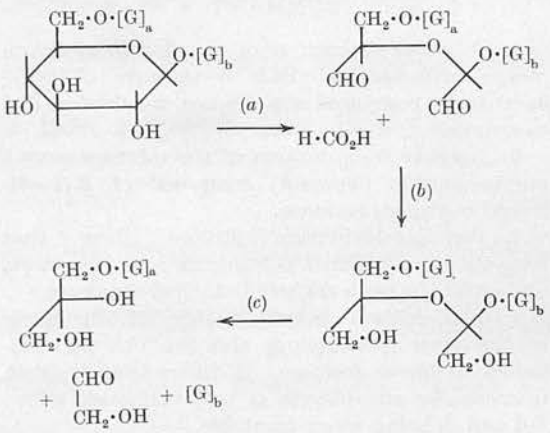
Nevertheless, additional periodate-oxidation experiments indicate that inter-chain linkages rather than internal (1→6)-glucosidic linkages are most probably present (Scheme 1).

Leucosin preparation III was subjected to the Smith degradation procedure (see Smith & Montgomery, 1959) involving periodate oxidation, borohydride reduction and partial acid hydrolysis. The residual polysaccharide was non-diffusible, and, on further oxidation with a dilute solution of sodium metaperiodate, the yield of formaldehyde was only one-fifth of that expected from structure (*c*), and one-tenth of that from structure (*b*). If leucosin contained internal (1→6)-glucosidic linkages, then the degradation procedure would yield linear chains of degree of polymerization 12–17. Since laminarin preparations with a degree of polymerization 20–25 are diffusible (W. D. Annan & D. J. Manners, unpublished work), any linear chains derived from leucosin should also be diffusible.

Moreover, partial hydrolysis of the leucosin polyalcohol would yield a 1-*O*-substituted glycerol (Scheme 1) that on controlled periodate oxidation would yield 1 mole of formaldehyde/molecule. The observed yield was only 0·204 mole/molecule, whereas in a control experiment with 1-*O*-β-glucosylmannitol 0·96 mole of formaldehyde/molecule was liberated. The small yield of formaldehyde could arise from a limited reaction at the residue of the original reducing group.

The fact that leucosin is not solely a linear polymer of β-(1→3)-linked D-glucose residues is also shown by the production of 0·5 mol.prop. of formaldehyde on overoxidation with periodate at pH 8. Although this value is similar to that obtained with laminarin (Anderson *et al.* 1958), the reasons for this are different. In laminarin, about 50 % of the polysaccharide chains are terminated by a 1-*O*-linked mannitol residue, and are therefore resistant to overoxidation. Mannitol is not a constituent of leucosin, and the observed yield of formaldehyde is consistent with a slightly branched structure.

The reserve carbohydrate of *Peranema trichophorum* (paramylon) resembles leucosin in several respects, but differs significantly in its solubility properties and in molecular size (degree of polymerization about 80). The relative insolubility of paramylon prevented a complete study of the extent of hydrolysis by the *Rhizopus* enzyme preparation and the extent of overoxidation by periodate (the yield of 0·3 mol.prop. of formaldehyde shows only the existence of adjacent (1→3)-linked glucose residues near to the reducing end of polysaccharide chain). The relative insolubility of long linear chains of β-(1→3)-linked glucose residues has also been noted with pachyman (Warsi &



Scheme 1. Degradation of an internal (1→6)-linked D-glucose residue by (*a*) periodate oxidation followed by (*b*) borohydride reduction and (*c*) partial acid hydrolysis. $[G]_a$ or $[G]_b$, chain of (1→3)-linked glucose residues.

Table 2. *Properties of some* β-(1→3)-*glucosans*

Property	Laminarin	Chrysolaminarin*	Paramylon	Leucosin	*Euglena gracilis* polysaccharide†
$[\alpha]_D$ (water) (°)	−9	−6	.	+10	.
$[\alpha]_D$ (NaOH) (°)	+9	.	+16	.	+28
Acetate, $[\alpha]_D$ (in chloroform) (°)	−60	.	.	−59	.
Infrared-absorption spectrum (absorption peak) ($cm.^{-1}$)	890	890	890	890	890
Periodate oxidation:					
at pH 5, reduction (molecule/anhydroglucose residue)	0·30	0·30	0·03	0·17	0·02
at pH 8, production of formaldehyde (molecule/anhydroglucose residue)	0·53	0·61	0·30	0·53	.
Hydrolysis by *Rhizopus* preparation	+	+	+	+	+

* See Beattie *et al.* (1961). † See Clarke & Stone (1960).

Whelan, 1957) and the paramylon from *Euglena gracilis* (Clarke & Stone, 1960).

The pattern of polysaccharide synthesis in *Ochromonas* is more complex than in *Peranema* since the former contains appreciable quantities of D-galactose and D-mannose-containing polysaccharide.

The results described in the present paper, together with those of Clarke & Stone (1960), provide the first evidence for the presence of β-(1→3)-linked glucose polymers in the protozoal group. This finding is of considerable biochemical interest since these linkages have hitherto been found only in polysaccharides isolated from yeast, marine algae, the fungus *Poria cocos* Wolf and callose-type membranes from certain higher plants (see Table 2 and, for example, Peat, Whelan & Edwards, 1958; Anderson *et al.* 1958; Warsi & Whelan, 1957; Aspinall & Kessler, 1957).

SUMMARY

1. Aqueous extraction of the flagellate *Ochromonas malhamensis* yields a mixture of polysaccharides composed of D-glucose, D-galactose and D-mannose.

2. Acetone fractionation of the mixture gave a polysaccharide (leucosin) composed of β-(1→3)-linked D-glucose residues.

3. Periodate-oxidation studies show that leucosin has a degree of polymerization of about 34, and probably has a slightly branched structure.

4. The reserve polysaccharide of *Peranema trichophorum* (paramylon) also contains β-(1→3)-linked D-glucose residues. It differs from leucosin in molecular size (degree of polymerization about 80) and in being water-insoluble.

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The Molecular Structure of a Reserve Polysaccharide (Paramylon) from *Astasia ocellata*.

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In a recent review of protozoal carbohydrates (Manners, D.J. & Ryley, J.F., 1963, *Biochem.J.*, 89, 88P) it was stated that the reserve polysaccharide (paramylon) from *Astasia ocellata* contained β-1,3-glucosidic linkages. We now report the experimental evidence for this view.

Paramylon was extracted from alcohol-dried cells, in 55% yield, using the procedure applied previously to *Peranema trichophorum* (Archibald, A.R., Cunningham, W.L., Manners, D.J., Stark, J.R., Ryley, J.F., 1963, *Biochem.J.* 88, 444). It was obtained in granular form, and was insoluble in cold and hot water, and in hot dilute sulphuric acid. The paramylon was soluble in N-sodium hydroxide and had $[\alpha]_D$ +17°; successive hydrolysis with hot 90% formic acid and 2N-sulphuric acid gave D-glucose in 96% yield. No other carbohydrate could be detected.

The presence of β-glucosidic linkages was shown by the slow degradation of paramylon by a β-glucosidase preparation from *Rhizopus arrhizus*, and by the infra-red spectrum which showed the expected absorption band at 890 $cm.^{-1}$

The presence of 1,3-linkages was suggested by the formation, on partial acid hydrolysis, of oligosaccharides with the paper chromato-

-graphic mobility of laminarisaccharides, and confirmed by the isolation of 2,4,6-tri-O-methyl D-glucose, in 96% yield, from an acid hydrolysate of the borohydride-reduced and methylated polysaccharide. The proportion of 2,3,4,6-tetra-O-methyl D-glucose (2.3± 0.1%) indicated an average chain length of about 43 glucose residues.

On periodate oxidation at pH5 and 27° for 72 hr., 0.14 mol. prop. of periodate was reduced per anhydroglucose residue; a linear chain of 43 β-1,3-linked glucose residues would reduce 0.12 mol. prop. The degree of polymerisation was estimated from the yield of formaldehyde produced on prolonged periodate oxidation, to be 52-55 glucose residues. This suggests that the paramylon has an essentially unbranched structure.

The reserve polysaccharide of A. ocellata is therefore similar to that synthesised by Ochromonas malhamensis, Peranema trichophorum (Archibald et al., loc. cit.), Euglena gracilis (Clarke, A.E. Stone, B.A., 1960, Biochim. Biophys. Acta, 44, 161) and Astasia longa (Picciolo, G.L. 1963, J. Protozool., 10, Suppl. 9).

The biosynthesis of paramylon is now being investigated; preliminary experiments by D.C. Taylor show that cell-free extracts of A. ocellata contain an enzyme, laminaribiose phosphorylase, which catalyses the formation of laminaribiose from α-D-glucose-1-phosphate and D-glucose.